SUBLETHAL EFFECTS OF POLYCHLORINATED BIPHENYLS (PCBs) ON BIRD BEHAVIOR

A Dissertation

Presented to the Facutly of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2012

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Cornell University 2012

Polychlorinated biphenyls (PCBs) are harmful, worldwide chemical pollutants. Most research on the biological effects of PCBs on wildlife has emphasized the lethal effects of PCBs. However, it is well known that PCBs have long lasting reproductive and endocrine effects at sublethal exposures. This thesis investigates the effects of sublethal PCB exposure on bird song, the song system, and song mediated behaviors in both field and captive settings.

In the field, black-capped chickadees (*Poecile atricapillus*) and song sparrows (*Melospiza melodia*) residing along the PCB-contaminated Hudson River in northeastern New York had higher blood PCB loads compared to individuals from control locations. PCB profiles of the two species varied substantially based on the proportion of lower chlorinated PCBs. High PCB levels correlated with significant, but contrasting effects on bird song structure. High loads were correlated with a greater proportion of black-capped chickadees singing inconsistent songs, and a greater proportion of song sparrows with high performance trills.

In captivity, the effects of Aroclor 1242 (the predominant commercial PCB-mixture originally contaminating the Hudson River) and PCB 52, an estrogenic congener, were investigated in zebra finches (*Taeniopygia guttata*). Results show that young male zebra finches exposed to sublethal PCB dosages learned songs similarly to those of control males, but the

treated males reproduce their song with less variability. In addition, PCB-exposure resulted in an increase in dendritic spines in the song motor area HVC, but no changes were seen in the robust nucleus of the arcopallium (RA).

Male zebra finches treated with PCBs had altered sexual behaviors, as well as other reproductive parameters, such as increasing the number of nesting attempts before a clutch successfully produces at least one hatchling, increasing the numbers of eggs laid before the first successful clutch, and increasing the number of eggs buried. Female zebra finches treated with Aroclor 1242 had stronger song preferences in a song choice assay.

The results of this thesis emphasize that sublethal PCB-exposure during development has subtle but important effects on adult passerine song and behavior, and these effects can have important repercussions to wild populations.

BIOGRAPHICAL SKETCH

Sara DeLeon was born in California in 1978. She graduated from the University of California, Davis in 2002 with a major in Biological Sciences, emphasizing in Neurology, Physiology, and Behavior, and minors in History and Mathematics. She also earned a four-year oar by rowing on the UCD women's crew team back when they were still DII. Sara gained her first research experience while she was at Davis. Working with Dr. Daniel W. Anderson, she studied the migration patterns of brown pelicans. She then went on to do a summer research program at the University of California, San Diego with Dr. Trevor Price and Dr. Pamela Yeh, studying song learning in dark-eyed juncos. These brief forays into research as an undergraduate opened up the realm of non-lecture-book learning to Sara, which had lasting consequences.

After Sara graduated from UC Davis she was tired of school. Determined to never again re-enroll she had a series of jobs spanning the truly horrible (detailing used cars) to the merely repetitive (working in the pharmaceutical industry). She managed to survive three years of job/life dissatisfaction before she reconsidered and applied to graduate schools. Only applying to out-of-state schools (at this point, Sara drastically needed a change of scenery), she didn't get into her back-up school of Kansas State, but was flabbergasted when she was admitted to Cornell. Figuring she ought to capitalize on their mistake, she packed her bags and moved across the country.

Drawing from the influential summer of fieldwork in San Diego as an undergraduate, Sara knew her graduate research interests were in 'the effects of anthropogenic disturbances on animal behavior'. Under the encouraging guidance of her advisor, Dr. André Dhondt, that phrase developed into the following dissertation.

ACKNOWLEDGEMENTS

The body of work represented in this dissertation would have been impossible to accomplish without the abundant encouragement, advice, and support from many individuals and groups.

First, and foremost, I want to thank my advisor, André Dhondt. From the very beginning, André encouraged me to pursue what I was interested in, regardless of whether it was his area of expertise or not. He trained me how to think and how to ask questions, and our discussions have broadened my perspective about my research. Through seven long years, André has discussed every aspect of this work with me and encouraged me every step of the way. The completion of this dissertation is evidence of the support he has shown me since we first met.

My committee has also played a central role in the completion of my dissertation. All the zebra finch work was done in the colony and lab of Tim DeVoogd. Tim's kindness and confidence in my abilities gave me the self-confidence to learn new techniques and take on extensive experiments. Mike Webster asked me great questions about my work, and I attribute many of the key ideas of this dissertation to his inquisitive mind. Jim Gillette (1933-2011) was a member of my committee for five years, and the 'PCB expert.' His advice about working with PCBs shaped this dissertation, and I am sorry he is not here to see its completion. And finally, prior to his retirement, Jack Bradbury was a member of my committee. His patience in teaching me details of birdsong and physics of sound is greatly appreciated.

All the PCB analysis was done in the lab of André Kessler, and I am grateful to André for allowing me to use his instrumentation. Yet even with the instruments, none of the PCB analysis would have been accomplished or possible without the expertise and genius of Rayko

Halitschke. I will never be able to thank him enough for his help, tenacity, and patience, both with the project and with me. I also want to thank Stefan Hames for his encouragement early on in my graduate studies, and for collaborating with me on the multiple stressors (specifically mercury and PCBs) that birds in eastern New York may be facing.

Financially, I was generously supported by a SLOAN fellowship, a Tibor T. Polgar fellowship, a SUNY fellowship, and a fellowship from the Cornell Lab of Ornithology. These monies allowed me to focus on the research and make substantial progress towards my degree. I am literally in your debt. Financial support for this research was also extensive. I received substantial support from Cornell in the form of Cornell Travel Grants, a Mellon Grant, and more than one Kieckhefer-Adirondack Grant. In addition, I received a BEB Small Grant at a key juncture in my time at Cornell, that I will always be grateful for. The bulk of the captive research was supported by a New York State Sea Grant (NYSG-081; PIs: André Dhondt and Tim DeVoogd). The support and interest of the NYSG personnel in this project and these questions was not only of great practical help, but also encouraging and taught me a great deal.

In the field, I would like to acknowledge the great many people that helped me gain access to land, set up field sites, and in general, just helped those four years of fieldwork be bearable. Dave Cerasale was integral in teaching me how to mist net and draw blood from passerines in the field. His expertise was invaluable to this work, and his attitude was invaluable to me. Garrett O'Connor at the NYS Canal System, Susan Wilder and Arthur Wright at the Hadley Town Park, Steve Lovering at Hudson Pointe, Charlotte Demers at the Huntington Wildlife Forest, Zev Ross in Ithaca, Patrick Landewe at the Saugerties Lighthouse, Robert Taylor at Schodack Island, Don and Nan Polunci and Pat Fitzgerald at the Southern Adirondack Audubon all helped with access and logistics at field sites.

For the captive experiments, I'd like to acknowledge Tim Van Deusen for his almost daily problem solving and troubleshooting. Elizabeth Adkins-Regan was a constant source of encouragement and timely advice. Otilia Menyhart helped with all aspects of zebra finch rearing. And Michi Schulenberg made the taxidermy mounts for the female choice experiment.

With the sound analysis, Greg Budney, Karl Fitzke, Dean Hawthorne, and Bill McQuay were instrumental in getting things to work and schooling me on sound equipment. Karl Berg, Becky Cramer, and Otilia Menyhart helped me with species-specific analysis questions. And the technical expert Brian Mlodzinski fixed my computer issues numerous times.

Steve Bogdanowicz first showed me the ropes of the genetic core facility, schooled me on procedures, and helped me figure out how to sex day old zebra finch nestlings. Nicole Baran was instrumental in helping with the microsatellites for parentage in the breeding experiment. Francoise Vermeylen and Wesley Hochachka gave excellent statistical advice. The Dhondt, Adkins-Regan, DeVoogd, and Webster lab groups saw numerous renditions of this research and gave insightful comments and suggestions. And many, many thanks to Stuart Campbell for applying his editing expertise to practically everything I have ever written.

I have been privileged to work with exceptional undergraduates while at Cornell. Their assistance with lab work, song analysis, and captive studies was of paramount importance. I am sincerely thankful to Camilo Acosta, Caleb Arellano, Adam Damon, David Dunstan, Anisleidy Fombona, Nana Fujiwara, Ben Gordon, Sophie Griswold, Elizabeth Liput, Chris Iriarte, Chris Stinson, and Natalie Zandt for their help throughout this project.

And lastly, I want to thank Harry Greene for convincing André that he should invite me to recruitment weekend in 2005. Thanks for seeing me for a bit more than what I look like on paper.

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Chapter 1:

INTRODUCTION

All organisms in the wild encounter environmental stress. This stress may be abiotic, such as food limitation (Hiom et al. 1991) and extreme weather conditions (Romero et al. 2000), or biotic, such as competition (Dhondt 2012) and predation (Bell et al. 2010). Although these 'naturally' occurring stressors are the focus of much ecological research, there is a growing number of studies that focus on the effects of anthropogenic disturbances on wildlife (Acevedo-Whitehouse & Duffus 2009; Adams 2003; Tarlow & Blumstein 2007). Research on anthropogenic disturbances to wildlife includes diverse topics such as urban noise (Slabbekoorn & Peet 2003), habitat fragmentation (Kruess & Tscharntke 1994), and chemical pollution (Zala & Penn 2004). This thesis focuses on the effects of a particular class of chemical pollutants, polychlorinated biphenyls (PCBs), and investigates effects of sublethal PCB exposure on passerine communication and behavior.

PCBs: Background information

PCBs are synthetic compounds first manufactured in the United States in 1929 for industrial purposes (Hutzinger et al. 1979). Their extensive uses in industry include heat transfer fluids, hydraulic fluids, solvent extenders, flame retardants, organic diluents, and dielectric fluids (Waid 1986). Although the manufacturing of PCBs was banned in the United States in 1977, they are serious environmental legacy chemical contaminants due to their widespread use, unregulated disposal practices last century, and longevity in the environment (Blais et al. 2006; Bonefeld-Jorgensen et al. 2001; Henshel & Sparks 2006). Not only do PCBs adhere to particulate matter

making them difficult to remove (Cho et al. 2001), they are also lipophilic, extremely stable, and slow to metabolize in biological systems (Waid 1986). It is estimated that one-third of the total PCBs manufactured in the United States have since entered the environment and are found in organisms at virtually every trophic level today (Brinkman & de Kok 1980; Clarkson 1995).

PCBs are composed of two benzene rings with between one and ten degrees of chlorination, allowing for 209 PCB congeners (stereochemical structures) with different toxicities, environmental persistence, and biological effects (Jofre & Karasov 2008; Khan & Thomas 2006; Tan et al. 2004). Commercially, PCBs were produced as congener mixtures, with specific percentages of chlorine by weight (Frame 1997). Therefore, organisms living in or near sites with environmental PCB-contamination almost always are exposed to multiple PCB congeners, congeners which each have distinct and diverse affects on biological systems.

Due to the structure and polarity of PCBs, once they are absorbed into the body, they are distributed to liver and muscle, and are stored in adipose tissue (Matthews & Dedrick 1984). Then the fate of a particular PCB molecule depends on the number and location of its chlorine substitutes. Non-*ortho*-substituted PCB congeners (no chlorines at the 2, 2', 6, 6' *ortho* position) have a flatter structure and act similarly to dioxin, another legacy environmental pollutant (Van den Berg et al. 2006). These dioxin-like PCBs act as agonists to the aryl hydrocarbon receptor (AhR) (Safe & Hutzinger 1987) and cause changes in the regulation of gene expression, increasing the synthesis of cytochrome P450 enzymes, a class of hepatic microsomal enzymes which catalyze the transformation of xenobiotic compounds to metabolites that are more easily eliminated from the body (Gillette et al. 1972). An increase in the number of cytochrome P450 enzymes is associated with many of the toxic effects of PCBs, including carcinogenesis (Safe & Hutzinger 1987). Non-*ortho* substituted PCBs with dioxin-like properties may also affect the AhR

and inhibit estrogen-responsive promoters in DNA or have direct affects on estradiol metabolism (Plíšková et al. 2005). The effects of *ortho*-substituted PCB-exposure are not as widely studied as non-*ortho*-substituted PCBs, yet recent studies do show that dendritic growth is altered with *ortho*-substituted PCB-exposure (Lein 2007; Wayman et al. 2012). In general, PCBs with more chlorine substitutes are longer lasting, less likely to be metabolized, and more toxic (Van den Berg et al. 2006). In contrast, PCBs with fewer chlorines are often associated with endocrine disruption (Crisp et al. 1998), with some congeners demonstrating estrogenic actions (Bonefeld-Jorgensen et al. 2001; Crews & Willingham 2000; Plíšková et al. 2005), while other congeners have an androgenic or antiandrogenic activity (Fang et al. 2003).

PCB exposure at high concentrations, regardless of the specific congener profile, can lead to acute effects such as embryo and juvenile mortality, slowed growth, cancer, and fatalities (Bertazzi et al. 1987; Henshel et al. 2006; Lavoie & Grasman 2007; Ruder et al. 2006). At sublethal concentrations, PCBs can alter sexual differentiation and the reproductive cycle in many organisms, such as reproductive failure in mink (Aulerich & Ringer 1977; Kihlstroem et al. 1992), altered sex ratios among the offspring of PCB-exposed turtles (Bergeron et al. 2008), and a lack of a normal ACTH-induced cortisol reaction to stress in fish (Hontela et al. 1992), to name a few. PCBs are known endocrine disruptors (Crisp et al. 1998) that act as exogenous agents and interfere with the action of natural hormones in the body. Because behaviors are the end point of neurological processes regulated by hormones, one expected consequence of sublethal PCB-exposure would be changes in behavior, although few studies have investigated this (Hoogesteijn et al. 2005; McCarty & Secord 1999a).

Bird song

Song is a fundamental component of bird communication and subsequent reproductive success (Kroodsma 1996). In most songbird species, song is learned early in development and is highly stable when produced in adulthood. One of the most basic functions of song is that it signals species identity. Many female passerine species use male song in selecting a mate, presumably because it is an honest indicator of quality (Searcy 1996). For example, male repertoire size has been shown to predict initial mating success and is correlated to fitness in male song sparrows (*Melospiza melodia*) (Reid et al. 2004, 2005). In addition, great tits (*Parus major*) that sing 'better' have higher individual lifetime reproductive success (Lambrechts & Dhondt 1986), while female canaries (*Serinus canaria*) prefer males that sing more complex songs (Spencer et al. 2005a). Males of many species also use song to define territories (Krebs et al. 1978). Thus, large changes in song characteristics could prevent conspecific recognition, and even slight changes could affect the establishment and defense of territories, mate attraction, pair bond maintenance, and ultimately, reproductive success.

The neurobiology of bird song has been intensively studied for more than 25 years (reviewed by DeVoogd and Lauay (2001)). The major nuclei in the song production pathway are the HVC (involved in organizing the song, particularly the syllables and their order, as a whole) that projects to the robust nucleus of the arcopallium (RA, involved in production of individual notes) that projects directly and via nucleus DM to the hypoglossal (nXII) nucleus, that in turn controls the muscles of the syrinx used for vocalizing (Figure 1.1). The HVC varies in size with song complexity, both across (DeVoogd et al. 1993) and within species (Airey & DeVoogd 2000; Canady et al. 1984; Reiner et al. 2004). In the song system each brain area has high levels of androgen and/or estrogen receptors (DeVoogd and Lauay 2001). The close integration of the

endocrine and neural systems suggests that any agent disrupting these endocrine receptors, such as PCBs, are likely to disrupt the behaviors organized by these regions. In particular, PCBs may be especially likely to cause such neural behavioral effects because of actions on steroid receptors and receptor function (Bonefeld-Jorgensen et al. 2001). Indeed, there is compelling evidence that RA volume decreases in progeny when zebra finches (*Taeniopygia guttata*) are exposed to a commercial PCB-mixture (Hoogesteijn et al. 2008). Although neurological damage is not necessarily lethal, these effects still could profoundly influence communication and other important reproductive parameters in songbirds.

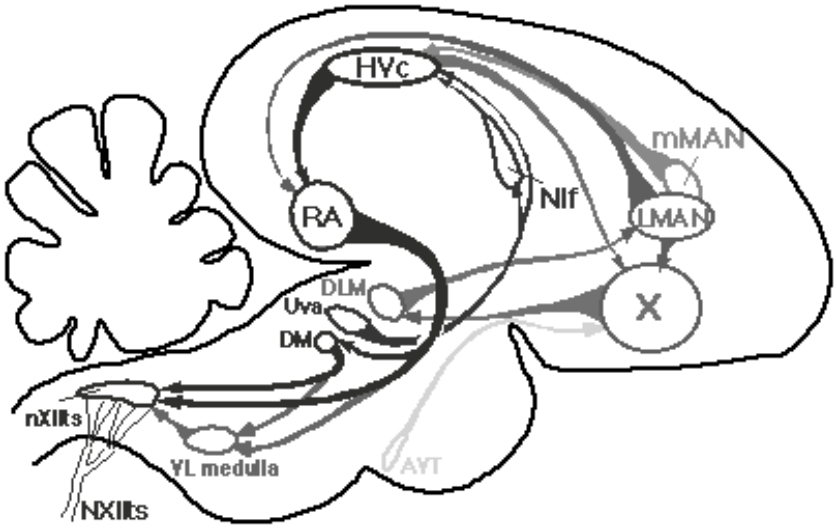

Figure 1.1. Sagittal view of the avian song nuclei. Schematic courtesy of Heather Williams.

Environmental chemical pollution and birdsong

Although few studies examine the effects of environmental chemical pollution on birdsong or song nuclei, those that have investigated the consequences reveal conflicting results. Dichlorodiphenyltrichloroethane (DDT), another persistent organochlorine compound, and its main metabolite *p, p′*-DDE, have been shown to cause decreases in HVC and RA volume in American robins (*Turdus migratorius*) (Iwaniuk et al. 2006). The authors did not measure impact

to the produced song, but did propose that the song nuclei results were an effect of stress, neurotoxicity, and androgen receptor antagonism (Iwaniuk et al. 2006). Heavy metal pollution (including mercury pollution) has been implicated in song changes in many species. Hallinger et al. (2010) recorded Carolina wrens (*Thryothorus ludovicianus*), house wrens (*Troglodytes aedon*), and song sparrows in areas of mercury pollution and found that these species produced shorter songs with lower note diversity and lower frequencies when compared to individuals at reference sites (Hallinger et al. 2010). Gorissen et al. (2005) found that great tits in areas with high levels of heavy metal pollution had smaller repertoire sizes and sang lower total amounts of song during the dawn chorus. Although neither sets of results from the DDT or heavy metal exposure studies investigate the impacts on both song and song nuclei, the studies do indicate that chemical pollutants can adversely impact aspects of song and song nuclei usually associated with male quality (DeVoogd et al. 1993; Pfaff et al. 2007). In contrast, Markman et al. (2008) reported that environmental estrogen exposure increased the HVC, song complexity, and female preference for song in European starlings (*Sturnus vulgaris*), although exposed males had reduced immune function. This is the first study I know of that looks at the impact of environmental pollutants on the cerebral song nuclei as well as the song characteristics. Furthermore, this study is the first to show that environmental pollutants can paradoxically improve song quality and increase song nuclei, while still compromising overall male quality.

I am aware of only one previous study that links PCB-exposure and song. When song nuclei volumes were measured in the progeny of Aroclor 1248-treated zebra finch (*Taeniopygia guttata*) mothers the RA was significantly smaller in both male and female finches (Hoogesteijn et al. 2008). Aroclor 1248 is a commercial mixture of PCBs with 48% chlorine mass by weight, and contains many higher chlorinated PCB congeners. Therefore, it seems that the developmental

exposure to these highly chlorinated PCB congeners results in song nuclei changes more akin to the studies with DDT.

Organization of this dissertation

This dissertation attempts to bridge a gap in the knowledge about the impact of sublethal PCB exposure on birdsong and song nuclei through both field studies and laboratory experiments. By recording and measuring songbirds in the wild, I determine whether individuals in areas with environmental PCB contamination display relevant song variations. In addition, by investigating the impact of environmentally relevant doses of a pure PCB congener and a commercial PCB mixture, I examine the consequences of different PCB congener profiles. I also consider endpoints of song other than just brain nuclei and song recordings by looking at song mediated behaviors, such as female song preference and male reproductive behavior.

Chapter 2 investigates PCB loads and songs of black-capped chickadees (*Poecile atricapillus*) and song sparrows along a PCB gradient at the Hudson River Superfund site in eastern New York State. Since most research on PCBs requires destructive sampling by measuring PCBs in tissues (Kania-Korwel et al. 2006; O'Keefe et al. 2006), one emphasis of this chapter was developing a non-destructive sampling method to characterize congener-specific PCB loads in both songbird species. Chapter 3 experimentally investigates the causal relationship between PCBs and birdsong. Captive zebra finches were dosed with environmentally relevant amounts of Aroclor 1242 or PCB 52 during development, and adult male song characteristics and song nuclei neuronal morphology were measured. Chapter 4 continues the investigation of the effects of Aroclor 1242 and PCB 52 on developing male zebra finches, and focuses on their reproductive output and behavior. The final data chapter, chapter 5, turns the focus to females,

and investigates the effects of developmental dosage of Aroclor 1242 and PCB 52 on adult body size, fledging age, and song preferences.

To date, no systemic research program has investigated behavioral consequences of PCB exposure, and relatively few studies have included any behavioral data (Zala & Penn 2004). Therefore, this dissertation attempts to add to our knowledge about the effect of PCBs on passerines, specifically exploring the impact of sublethal PCB-exposure on birdsong and reproductive behavior.

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Chapter 2:

IMPACT OF POLYCHLORINATED BIPHENYLS (PCBS) ON BIRDSONG

Abstract

During the last century, polychlorinated biphenyls (PCBs) have emerged as harmful chemical pollutants worldwide. Although the critical toxic effects of PCBs on wildlife are well-documented, little is known about the behavioral consequences of exposure to sublethal PCB levels. PCBs are predicted to influence a range of behavioral traits due to their effects on endocrine and neurological systems governing those behaviors. We investigated PCB loads and songs of black-capped chickadees (*Poecile atricapillus*) and song sparrows (*Melospiza melodia*) along a PCB gradient at the Hudson River Superfund site in New York State. We developed a novel non-destructive sampling method and characterized congener-specific PCB profiles for both songbird species. In addition, we quantified body condition and blood mercury concentration to account for other factors that possibly influence song quality in this region. Our results indicate that regions with higher historic PCB contamination have black-capped chickadees and song sparrows with higher blood PCB loads. PCB profiles of the two species varied substantially based on the proportion of lower chlorinated PCBs. High PCB levels had significant, but contrasting effects on bird song structure. High loads were correlated with a greater proportion of black-capped chickadees singing inconsistent songs, but a greater proportion of song sparrows with high performance trills. Body condition and blood mercury concentrations results did not adequately explain these song patterns. Thus, PCBs can alter a vital component of communication in birds, and the ramifications of changes in song quality for bird populations extend the toxic effects of environmental PCB pollution.

Introduction

Polychlorinated biphenyls (PCBs) are global chemical pollutants first manufactured in the United States in 1929 for industrial purposes such as heat transfer fluids, flame-retardants, and organic diluents (Leigh et al. 2006; Waid 1986). Although PCBs were banned in 1979, they continue to pose serious environmental risks due to their widespread application, unsound disposal practices, and chemical characteristics (Blais et al. 2006; Henshel & Sparks 2006). Worldwide, approximately 31% of all PCBs manufactured are still present in the environment, and only an estimated 4% are degraded or destroyed (Crisp et al. 1998).

PCBs are a complex class of chemicals. The two benzene ring structure allows for ten possible positions of chlorination, resulting in 209 congeners with variable toxicities, environmental persistences, and biological effects (Jofre & Karasov 2008; Khan & Thomas 2006; Tan et al. 2004). In general, PCBs with more chlorine substitutes are longer lasting, less likely to be metabolized, and more toxic (Van den Berg et al. 2006). Coplanar PCBs (unchlorinated at the 2, 2', 6, 6'-*ortho* position) or mono-*ortho*-substituted PCBs have a flatter structure with chemical properties and toxicities similar to dioxin (Van den Berg et al. 2006). PCBs are also endocrine disruptors (Ottinger et al. 2008), and PCBs with fewer chlorines are associated with estrogenic actions (Bonefeld-Jorgensen et al. 2001; Crews & Willingham 2000; Plíšková et al. 2005), while some congeners have demonstrated androgenic or antiandrogenic activity (Fang et al. 2003). All PCBs are lipophilic, extremely stable, and slow to breakdown and metabolize in biological systems (Waid 1986).

Because the location and the number of chlorines on the di-benzene backbone dictate the toxicity and chemical fate of PCB congeners, specific congener data (in contrast to total PCB concentration) is necessary to understand the possible effects of PCBs in living organisms.

However, PCB profiling is often only performed on larger organisms at high trophic positions, because PCBs bioaccumulate and biomagnify (Cogliano 1998; Waid 1986), and because the large size of the sample permits analysis of congener-specific PCBs (see (Bustnes et al. 2005; Fasola et al. 1998) for examples). When PCBs are measured in smaller organisms at lower trophic positions, destructive sampling is commonly used to obtain samples large enough in size to determine congener-specific PCB data (e.g., (Henshel & Sparks 2006; Neigh et al. 2006)). In circumstances where species are protected or endangered, the alternative is to look at PCB contamination levels in carcasses of individuals that have 'naturally' died (Senthil Kumar et al. 2002), a practice that may result in biased sampling for PCB levels. Although advances have been made to develop new methods to non-destructively measure PCB load in small amounts of wildlife blood, or feather and hair samples (Covaci & Schepens 2001; Dauwe et al. 2005; Jaspers et al. 2007; Rivera-Rodriguez et al. 2007), these methods have not yet been commonly tested or used in field studies.

PCB congeners are known to interfere with natural endocrine regulation (Crisp et al. 1998). Since most complex behaviors are the endpoints of neurological processes regulated by hormones, it has been hypothesized that PCBs could have widespread behavioral effects (Dell'Omo 2002). Yet, there are few empirical studies supporting this hypothesis (McCarty & Secord 1999a). Establishing the magnitude and extent of PCB effects on behavior would provide a framework for assessing, predicting, and mitigating the consequences of sublethal PCB exposure for sensitive wildlife populations.

Song is a critical behavior for many bird species and is used primarily for mate identification and attraction, and territory defense (Brumm et al. 2011). We hypothesized that birdsong would be sensitive to the effects of PCBs, for both ecological and physiological reasons.

First, songbirds foraging in contaminated riparian zones are ingesting insects which contain high levels of pollutants (Park et al. 2009). Second, androgenic and estrogenic metabolites of testosterone are important in regulating the song control nuclei in the passerine brain (reviewed in (Ball et al. 2002)), and disruption of this regulation by PCBs should alter song phenotypes. Third, the robust nucleus of the arcopallium (RA), a region in the neurological song system in bird brains, decreases in volume with PCB exposure (Hoogesteijn et al. 2008). Since the volume of nuclei in the song system are positively correlated with aspects of song quality (Adkins-Regan 2005; Canady et al. 1984; DeVoogd et al. 1993; Reiner et al. 2004; Tramontin et al. 1999), PCBs could directly influence the neural basis of song characteristics.

The purpose of this study was to investigate whether sublethal levels of PCBs affect biologically relevant birdsong characteristics in natural populations. We focused on black-capped chickadees (*Poecile atricapillus*) and song sparrows (*Melospiza melodia*) along a PCB gradient in northeastern New York State, in order to: 1) non-destructively sample blood from passerines in the field to quantify congener-specific PCB levels and 2) determine changes to bird song characteristics as a function of PCB exposure. To account for additional environmental and developmental factors that may influence bird song characteristics in these regions, we also quantified blood mercury concentrations (Lorey & Driscoll 1999) and body condition of the two species (Galeotti et al. 1997).

Methods

Study sites

The Hudson River basin in northeastern New York contains high PCBs levels that vary throughout the drainage (Echols et al. 2004). From 2006 to 2009, 47 recording sites, and 7 field

sites were established in five regions in New York State (Figure 2.1). These sites were chosen based on documented variability in the levels of PCBs found in the Hudson River basin (EPA 1997). Regions 1 (N=5) and 2 (N=14) are outgroups, with no known PCB contamination. Region 3 (N=5) is a control site above the point source of pollution, with low documented PCB levels (EPA 1997). Region 4 (N=12) is directly below the pollution point source at Fort Edward and Hudson Falls, with the highest PCB contamination (EPA 1997). Region 5 (N=12) is downstream of the dam at Troy, with an intermediate PCB level (EPA 1997). The sites in Regions 1 and 2 were within 40m of a river or lakeshore, and the sites in Regions 3-5 were within 40m of the Hudson River. All sites were in, or on the edge of mixed deciduous/evergreen forests, with similar amounts of tree cover.

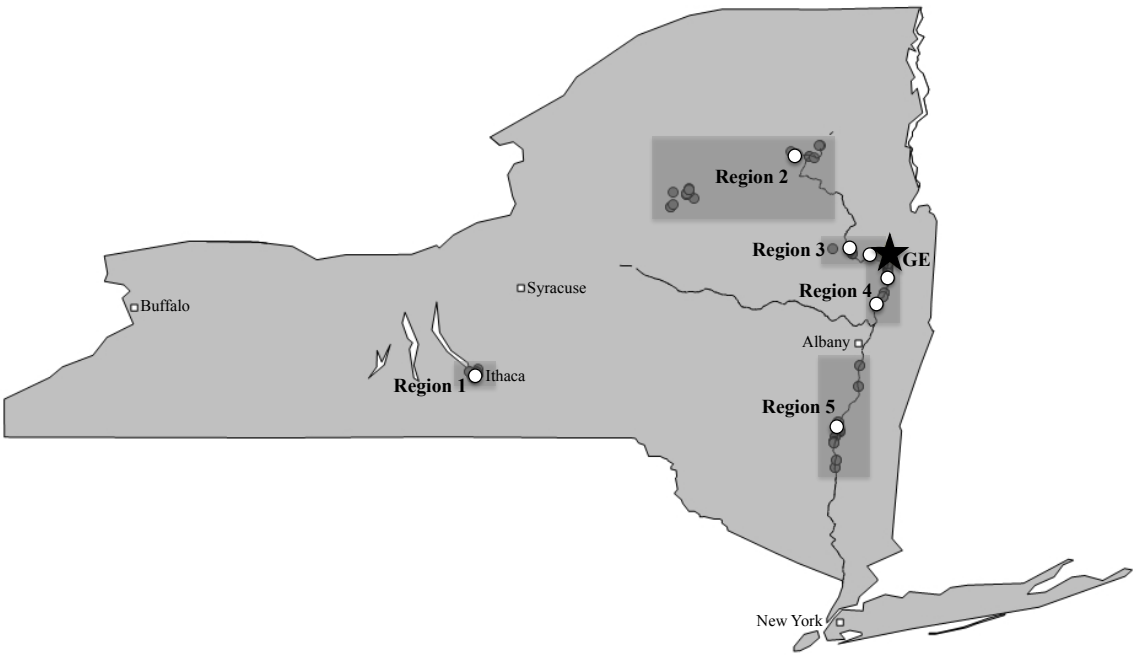

Figure 2.1. Map of recording and sampling sites. The five study regions are indicated by grey rectangles. Region 1 is in Ithaca, NY. Region 2 is in the Adirondacks. Region 3 is directly above the point source pollution at General Electric (GE, indicated by a star), Region 4 and 5 are downstream of GE. Recording sites are indicated with dark gray points, and sampling sites are indicated with white points.

Study species

We used the black-capped chickadee and the song sparrow as study species because both have life history traits and song characteristics that make them particularly amenable to PCB exposure. Adults feed their young insects, and also primarily eat insects themselves during the breeding season (Bent 1964; Judd 1899). Males have territories within foraging distance to water (Smith 1991; Stauffer & Best 1980), are either year-round residents or have high between-year site fidelity, and have short natal dispersal distances (Nice 1937; Robbins et al. 1986). Because insects with an aquatic larval stage have higher amounts of pollutants if collected near a pollution source (Park et al. 2009), it is reasonable to infer that birds born in areas with high PCBs are likely to be ingesting them for their entire life. In addition, black-capped chickadees and song sparrows both have well studied songs and specific song characteristics that identify high quality males. The glissando and interval ratios were used as a measure of male quality for black-capped chickadees (Figure 2.2a), and the trill-rate frequency-bandwidth trade-off was used as a measure of male quality for song sparrows (Figure 2.2b) (Horn et al. 1992; Podos 1997; Weisman & Ratcliffe 2004).

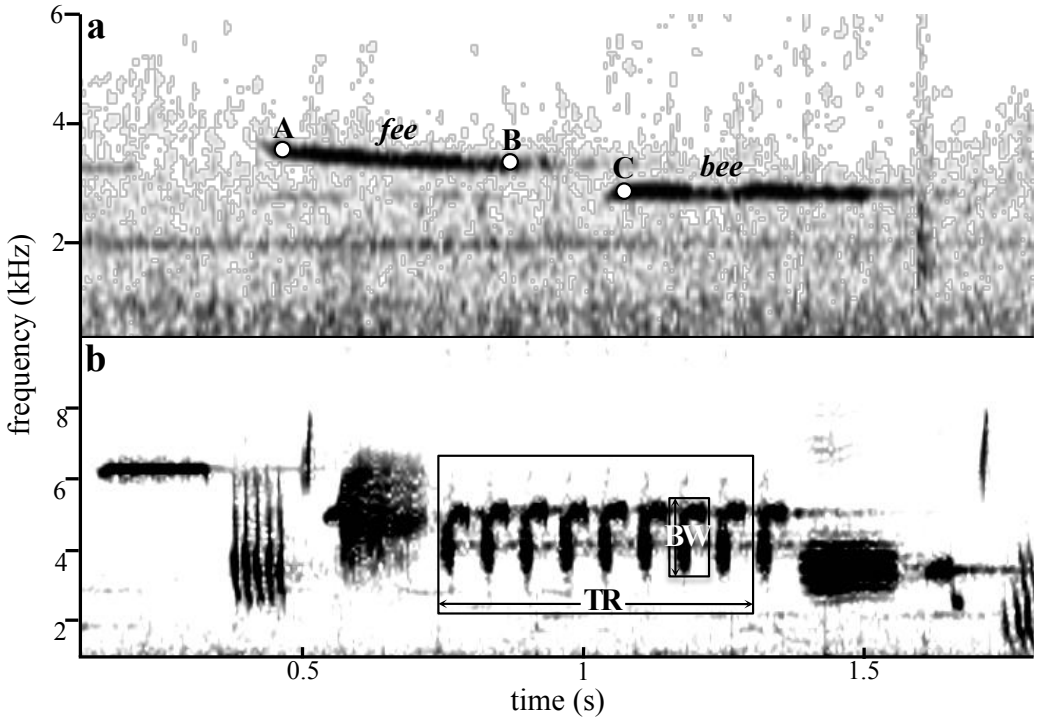

Figure 2.2. a) Spectrogram of a male black-capped chickadee *fee-bee* song. The glissando ratio is the frequencyA/frequencyB. The interval ratio is the frequencyB/frequencyC. **b)** Spectrogram of a male song sparrow song type. The trill rate (TR) is the number of syllables (eight in this song)/unit time. The bandwidth (BW) is the span of frequencies (high-low) of a syllable.

Mist netting

In May and June 2008-09, black-capped chickadees and song sparrows were captured using 6-foot or 12-foot 30mm mist nests (Avinet, Inc. Dryden, NY 13053 USA; USFWS bird banding permit 22669: André A. Dhondt). To attract the birds to the mist nets, birdfeeders with sunflower seeds were hung near net locations. Playbacks with recordings from the target species were played directly adjacent to the nets to attract territorial males. The playbacks were all songs from wild individuals recorded during the two previous field seasons (2006-2007) at comparable sites. All sampling and field measurements were performed on birds within 20 minutes of capture and all individuals were released directly following the procedure and flew away with no apparent

distress. All bird-handling procedures were approved under Cornell Universities Institutional Animal Care and Use Committee (Protocol Number: 2008-0022).

Species were identified and sexed according to Pyle (1987), and then banded with metal USFWS bands. Wing chord, tarsus length, weight, body condition index (score from 0-5 of the size of the pectoral muscle), fat score (score from 0-3 of the stores in the furcula) and mites (score from 0-3 of ectoparasite damage on the primary wing feathers) were recorded.

Blood samples were collected, when possible, from all individuals. Approximately 100 μL of whole blood was taken from the left brachial vein with a 27" gauge single-use needle (BD Medical, length: 12.7mm). The blood samples for PCB analysis were collected with a non-heparinized capillary tube and immediately transferred to 1.5 mL centrifuge tubes (EDTA washed to prevent coagulation) and stored on ice. PCB samples were separated into red blood cells and plasma with a Mini Centrifuge (Fisher Scientific, Inc., 2000 Park Lane Dr. Pittsburgh, PA 15275 USA) in the field and flash frozen on dry ice or liquid nitrogen and stored at -80°C for later analysis. The blood samples for mercury analysis were collected with a heparinized capillary tube, then stored on ice as whole blood until they were transferred to a -20°C freezer until analysis.

PCB blood analysis

Blood serum samples from black-capped chickadees (22.1±2.2mg) and song sparrows (50.6±2.2mg) were fortified with 25μL of $^{13}C_{12}$-labeled internal standard mixture (EC-5087, Cambridge Isotope Laboratories, Inc., Andover, MA, USA). Samples were extracted twice with 100μL of n-hexane as described by Rivera-Rodriguez et al. (2007). The hexane extracts were dried over 100mg anhydrous sodium sulfate and the volume reduced to 50μL under a gentle stream of nitrogen. PCB levels were determined by gas chromatography mass spectrometry

(GCMS) on a Varian Saturn 2200 ion trap GCMS system (Varian, Inc., Walnut Creek, CA). One μL aliquots were injected into the GC at an injector temperature of 300°C. PCBs were separated on a VF-5MS capillary column (30m x 0.25mm ID, 0.25 micron film thickness; Varian, Lake Forest, CA) with helium as carrier gas at a flow rate of 1mL min^{-1} and the following temperature gradient: 40°C for 1 min, at 30°C min^{-1} to 160°C, at 4°C min^{-1} to 220°C, at 12°C to 300°C min^{-1}, and 300°C for 5min. Congener-specific selected reaction monitoring (SRM) conditions were optimized for the quantitation of 41 of the 48 most abundant congeners reported for the Hudson river Superfund site (Trustees 2005) with quantitation limits of 0.1 to 1ppb. Calibration curves were generated using a dilution series made from pure PCB standards (AccuStandard, Inc., New Haven, CT, USA).

Mercury blood analysis

Blood samples were analyzed for total mercury content (THg) at the Center for Environmental Sciences and Engineering (University of Connecticut, Storrs, CT, USA) using Environmental Protection Agency Method 1631. Blood was first digested with sulfuric and nitric acids, oxidized using bromine monochloride, and trapped on a gold trap. The trap was purged into cold vapor atomic florescence (CVAFS) unit for analysis. Detection limit for blood THg was 0.02 ng/g. Standard quality assurance methods were used: analysis of duplicate samples; method blanks; spiked samples; laboratory control samples; and standard reference materials (DOLT-3, DORM-2, 966). Instrument response was evaluated using a calibration verification standard and blank, before, during (every 20 samples), and after each analytic run.

Song recording

All recordings were made with a Fostex FR-2 Field Memory Recorder or a Tascam HD-P2 Recorder, a Universal Telinga Pro 24-inch Parabola, and a Sennheiser ME62 Omni

Microphone. Recordings were performed with a sampling width of 24 bits and a sampling rate of 44.1 kHz. All songs were recorded between 0400 and 1000 EST, from April to August in 2006, 2007, and 2009. Recordings were made of spontaneous male black-capped chickadee and song sparrow song. To increase the likelihood of non-repeated sampling, individuals were distinguished by either visual or auditory cues. If there still was uncertainty of replication, the next recording was made at least one territory's distance away (~160m). In 2009, a subset of the song sparrows that were blood sampled were also color banded and recorded.

Song analysis

The familiar *fee-bee* song (Figure 2.2a) is usually only sung by male black-capped chickadees in a territorial context and to attract and arouse females during the breeding season (Weisman & Ratcliffe 2004). The glissando ratio is thought to indicate species identity, and varies <2% over the species range (Christie et al. 2004; Weisman et al. 1990). The interval ratio is thought to be an indication of male quality, with higher quality males able to reproduce the ratio consistently at all frequencies (Christie et al. 2004). The interval ratio also varies <2% over the entire black-capped chickadee range (Christie et al. 2004).

Only black-capped chickadees recorded singing at least eight consecutive non pitch-shifted *fee-bee* songs were used in this analysis (N=235). Spectrograms were generated in RavenPro 1.4 (Bioacoustics Research Program, Cornell University) with a 512 sample size, a 512 DFT size, spectral overlap of 50% (Hann window), 3dB filter bandwidth at 135 Hz, 256 sample hop size, grid spacing of 93.8 Hz, no clipping, and averaging 1 spectrum. The glissando ratio was calculated by dividing the 95% *fee* frequency by the 5% *fee* frequency. The 95% *fee* frequency is the frequency value for a selected *fee* note where 95% of the energy is below that value. Similarly the 5% *fee* frequency is the frequency value for a *fee* note where 5% of the energy is below that

value. These two measurements calculated by RavenPro give a stereotyped frequency value of the beginning *fee* note frequency and the end *fee* note frequency. The interval ratio was calculated by dividing the center frequency of the end of the *fee* note by the center frequency at the beginning of the *bee* note. Because a stereotyped measurement was not possible to use for the interval ratio, I blindly made all the interval ratio measurements, for consistency. To look at the variation of the song structure within an individual black-capped chickadee, the coefficient of variation (CV) of the glissando ratio and the interval ratio was calculated for each individual. Individuals with inconsistent songs were quantified as birds that had a glissando ratio or interval ratio CV value greater than 0.03. Individuals with consistent songs sang the ratios with a CV less than 0.03. We choose 0.03 as the cut-off point because it represented a natural break in the CV values in the data throughout all regions. Since consistency in the glissando and interval ratios is used as a measure of song quality, individuals' singing with a CV greater that 0.03 was a conservative measure of the individuals singing inconsistent songs.

Song sparrows are known for their melodic and varied songs. Territorial males typically sing a repertoire of 5-13 song types (Figure 2.2b), during the breeding season (Cassidy 1994; Hiebert et al. 1989; Kramer & Lemon 1983; Podos et al. 1992; Searcy 1984). Although large repertoire size has been linked to longer territory retention, higher reproductive rates, and an increased initial mating success (Hiebert et al. 1989; Reid et al. 2004), accurate estimates of repertoire size are difficult to obtain (minimum of 200 recorded songs needed (Hughes et al. 1998)). Therefore, a more feasible metric used to classify song sparrow song performance is the trill-rate frequency-bandwidth trade-off (Podos 1997). The trill is a phrase present in most song sparrow song types, characterized by the rapid repetition of a syllable. The trill rate is the number of syllables produced per unit time (Hz), and the frequency bandwidth is the difference between

the maximum and minimum frequencies of the syllables in the trill. The trade-off between these two measurements, the trill rate and the frequency bandwidth, makes intuitive sense. The bird is constrained in how quickly it is able to transition between low frequency production (with a relatively closed beak and a fully inflated nasopharyngeal cavity) and high frequency production (with a relatively open beak and an only partially inflated nasopharyngeal cavity).

Song sparrows that sang song types containing a trill were included in the analysis if they sang a minimum of five repetitions of the trill, and if the trill contained a minimum five syllable repetitions (N=155). Spectrograms were generated in RavenPro 1.4 (Bioacoustics Research Program, Cornell University) with a 557 sample size, a 1024 DFT size, spectral overlap of 80.1% (Hann window), 3dB filter bandwidth at 124 Hz, 111 sample hop size, grid spacing of 46.9 Hz, no clipping, and averaging 1 spectrum. The trill rate of a trill of *n* syllables was calculated by dividing the count of *n-1* syllables by the time span of *n-1* syllables (to account for inter-syllable time). The bandwidth was measure by subtracting the 0.5% frequency from the 99.5% frequency, and averaged for at least five syllables per trill. Trills were binned into 5-Hz trill rate increments, and from each bin, the trill with the maximum frequency bandwidth was selected and used to produce the linear regression (Podos 1997). Individuals singing trills with a trill rate and a bandwidth that fall close to the regression line are singing high performance (low deviation) trills and individuals singing trills with a trill rate and a bandwidth that fall far from the regression line are singing low performance (high deviation) trills. Since trill types varied greatly within and between regions, no attempt was made to classify the trill types, and all trills were pooled for analysis. This greatly increases the noise of the data, decreases the likelihood of finding any pattern, and makes this a conservative analytical approach. Many male song sparrows were recorded singing multiple trill types, therefore one trill type was randomly chosen for each male

to avoid psuedoreplication. Individuals with high performance (low deviation) trills were quantified as birds that sang a trill with a distance less than 4 from the regression line. Individuals with low performance (high deviation) trills had a distance greater than 4 from the regression line. Four was chosen as the classification parameter because it resulted in the most conservative grouping.

Statistical analysis

Total PCB data was log-transformed and analyzed with a two-way ANOVA where species identity and region were random effects, followed by a Tukey post hoc test. Congener specific PCB data was analyzed in Region 4 with a principle components analysis (PCA). The proportions of tetra- and penta-chloro (4-Cl+5-Cl), and hepta- and octa-chloro (7-Cl+8-Cl) PCBs in Region 4 were compared separately between the two species with Student's *t*-tests.

The regional differences of black-capped chickadee glissando ratios were analyzed with the Kruskal-Wallis test, and the Wilcoxon test with a Bonferroni correction ($P<0.05$) was used post hoc to determine statistical differences between the regions. The regional differences of black-capped chickadee interval ratios were analyzed with an ANOVA where region was the fixed effect. A Chi-square test was used to test whether proportions of inconsistent and consistent individual black-capped chickadee singers differed between regions. A Chi-square test was also used to test whether proportions of individual song sparrows singing high performance and low performance trills differed between regions.

Results

PCB concentrations in black-capped chickadee and song sparrow blood

Analysis of congener-specific PCB profiles in the blood of black-capped chickadees and song sparrows along the contamination gradient in northeastern New York (Figure 2.1) revealed region- and species-dependent differences in PCB concentration and congener composition. The average concentration of the 41 congeners analyzed for black-capped chickadees (Table 2.1) and song sparrows (Table 2.2) varied greatly among the five regions and are comparable to previously reported values (Secord et al. 1999). Total PCB concentration was highest in Region 4 (Figure 2.3a), which is historically the area of highest PCB contamination (EPA 1997). In Region 1 and 3 the two bird species had a similar congener profile (Figure 2.3b). In Region 2, chickadees had higher penta- and hexa- chlorinated PCB levels, while the song sparrows had more evenly distributed levels of PCB chlorination. The greatest difference in PCB content of the two species was in Region 4, directly below the point source pollution from the General Electric (GE) plant. To examine the different congener profiles between the chickadees and sparrows in Region 4 in more detail, a Principal Components Analysis (PCA) was performed on the PCB congener data (Figure 2.3c). The difference in congener profiles for chickadees and sparrows partitioned clearly along PC2, which corresponds to a higher proportion of congeners with a lower degree of chlorination based on factor loading. Chickadees contained a non-significant, but slightly higher proportion of hepta- and octa- chlorinated congeners (Figure 2.3d; Student's *t*-test: t=1.30, *P*=0.20), while song sparrows had a significantly higher proportion of lower chlorinated PCBs (4-Cl+5-Cl) than chickadees (Figure 2.3d; Student's *t*-test: t=-3.36, *P*=0.0016). Similarly, the ratio of high to low chlorinated PCBs was higher in chickadees and lower in song sparrows in Region 5, downstream from the point source of pollution (Figure 2.3d; Student's *t*-test: t=-2.66, *P*=0.01).

Table 2.1. Individual PCB congener concentrations. Concentrations are shown as average (ppb) ±SE for black-capped chickadees, by region.

		Black-capped Chickadees				
	Region	**1**	**2**	**3**	**4**	**5**
PCB Congener	**8**	0±0	0±0	0±0	0±0	0±0
	18	0±0	0±0	0±0	0±0	0±0
	28/31	0±0	0±0	0.059±0.043	3.241±0.86	0±0
	44	0±0	0±0	0±0	0.122±0.094	0±0
	45	0±0	0±0	0±0	0±0	0±0
	47	0±0	0±0	0.132±0.085	18.104±2.592	0.973±0.487
	49	0±0	0±0	0±0	8.208±1.783	0.183±0.183
	52	0±0	0±0	0±0	16.975±2.986	0.577±0.31
	56/60	0.21±0.21	0.197±0.197	1.226±0.549	3.938±0.996	1.96±1.96
	66	0.196±0.196	0±0	1.553±0.428	18.311±3.285	0.897±0.491
	70	0.396±0.246	0.25±0.25	1.392±0.574	0.759±0.247	0.197±0.197
	74	0.246±0.215	0±0	1.779±0.577	16.191±3.265	2.897±1.128
	77	0±0	0±0	0.257±0.257	0±0	0±0
	81	0±0	0±0	0±0	0±0	0±0
	87	0±0	0±0	0±0	1.105±0.483	0±0
	95	2.342±1.609	2.103±1.112	7.784±4.704	0±0	1.827±1.827
	105	0±0	0±0	1.065±0.794	12.133±2.172	0±0
	110	0±0	0±0	0±0	0.377±0.377	0±0
	114	0±0	0.885±0.885	0±0	1.39±0.584	0±0
	118	0±0	0±0	2.386±1.173	30.018±7.899	2.317±2.317
	123	0.196±0.196	0±0	0±0	1.629±0.494	0±0
	128	0±0	0±0	0.059±0.059	9.628±2.962	0.553±0.279
	132/153	2.436±0.819	1.09±0.37	9.457±2.861	82.425±15.824	35.843±6.634
	146	0±0	0±0	1.159±0.413	14.195±2.755	6.417±1.27
	149	0±0	0±0	0.218±0.12	5.594±1.241	0±0
	151	0±0	0±0	0.038±0.038	0.368±0.216	0±0
	156	0±0	0±0	0.148±0.148	4.746±1.313	1.083±0.545
	157	0±0	0±0	0±0	1.108±0.431	0±0
	167	0±0	0.617±0.617	0±0	1.723±0.907	0±0
	169	0±0	0±0	0±0	0.254±0.254	0±0
	170	3.26±1.777	0.345±0.345	2.831±0.843	12.391±3.105	5.497±0.924
	174	0.288±0.288	0±0	0.207±0.131	1.555±1.221	1.11±0.681
	177	1.16±1.16	0±0	0.077±0.06	1.26±0.952	0±0
	180	2.06±1.466	0.525±0.525	5.891±2.641	41.49±15.011	22.767±4.168
	183	1.88±1.491	0±0	1.988±1.039	16.779±9.478	4.1±3.489
	187	0.334±0.334	0.665±0.665	0.96±0.75	19.227±19.227	0±0
	194	0±0	0±0	0.686±0.579	5.195±1.344	0.833±0.833
	195	0±0	0±0	0±0	0.558±0.373	0±0
	206	0.974±0.601	0.34±0.34	0.719±0.248	3.124±0.774	1.827±0.982
	209	0±0	0±0	0±0	0±0	0±0
	Total	15.98±5.432	5.843±2.74	41.578±9.455	354.122±74.389	91.85±2.816
	N	5	3	18	10	3

Table 2.2. Individual PCB congener concentrations. Concentrations are shown as average (ppb) ±SE for song sparrows, by region.

		Song Sparrows				
	Region	**1**	**2**	**3**	**4**	**5**
PCB Congener	**8**	0±0	0±0	0±0	0±0	0±0
	18	0±0	0±0	0±0	0±0	0±0
	28/31	0±0	0±0	0.723±0.207	89.838±18.257	4.427±0.842
	44	0±0	0±0	0±0	0.071±0.047	0±0
	45	0±0	0±0	0±0	0±0	0±0
	47	0.254±0.13	0.171±0.072	0.741±0.134	121.378±21.586	5.997±0.929
	49	0±0	0±0	0.031±0.031	26.247±4.586	0.568±0.144
	52	0±0	0±0	0±0	22.021±4.317	0.288±0.122
	56/60	0±0	0±0	0.263±0.087	36.893±8.839	0.536±0.153
	66	0.736±0.315	0.57±0.214	3.293±0.663	183.613±37.349	5.592±0.752
	70	0±0	0±0	0±0	13.684±2.385	0.284±0.153
	74	0.569±0.234	0.448±0.113	1.92±0.359	112.947±23.616	3.988±0.589
	77	0±0	0±0	0±0	0.281±0.084	0.095±0.055
	81	0±0	0±0	0±0	0.113±0.082	0±0
	87	0±0	0±0	0±0	0.812±0.595	0±0
	95	0±0	0±0	0.138±0.138	0±0	0±0
	105	0±0	0.445±0.319	1.653±0.359	66.555±13.222	3.589±0.488
	110	0±0	0±0	0±0	10.731±2.965	0.739±0.244
	114	0±0	0.165±0.165	0.616±0.27	7.936±1.411	0.771±0.273
	118	0.25±0.25	1.267±0.654	3.242±0.571	98.471±17.874	6.801±1.217
	123	0.088±0.088	0.055±0.055	0.137±0.053	9.203±1.752	0.508±0.161
	128	0.044±0.044	0.056±0.056	0.642±0.175	13.953±3.034	2.279±0.566
	132/153	1.54±0.356	2.462±0.233	8.498±1.327	139.162±20.633	11.486±3.671
	146	0±0	0±0	1.178±0.266	23.431±3.302	1.835±0.583
	149	0±0	0±0	0.036±0.036	8.597±1.472	0.702±0.21
	151	0±0	0±0	0±0	0.255±0.093	0±0
	156	0.106±0.073	0±0	0.297±0.089	4.773±0.663	0.563±0.141
	157	0±0	0±0	0±0	2.003±0.421	0.083±0.058
	167	0±0	0.104±0.104	0.422±0.231	2.456±0.632	1.833±0.674
	169	0±0	0±0	0±0	0.035±0.035	0.046±0.046
	170	0.576±0.393	0.49±0.244	2.473±0.657	25.404±5.562	8.979±2.207
	174	0±0	0±0	0.027±0.027	0±0	0.219±0.102
	177	0.023±0.023	0±0	1.398±0.412	16.8±5.708	0.91±0.342
	180	2.552±1.019	1.491±0.483	9.424±1.99	94.144±23.971	18.731±4.489
	183	0.691±0.366	0.734±0.366	4.284±1.418	42.868±15.159	3.455±1.173
	187	0±0	0.107±0.107	0±0	0±0	2.745±0.698
	194	0.887±0.435	0.797±0.293	1.616±0.425	5.119±0.569	2.1±0.381
	195	0.729±0.426	0.635±0.407	0.568±0.195	1.749±0.322	1.303±0.415
	206	0.785±0.528	0.646±0.247	0.772±0.191	8.007±1.603	3.442±0.67
	209	0±0	0.742±0.742	0±0	0.168±0.168	0.156±0.156
	Total	9.831±2.788	10.835±1.444	42.294±6.359	1150.065±185.934	95.011±13.712
	N	14	11	29	39	32

Table 2.3. The limit of quantification (LOQ) and the limit of detection (LOD) are given for each analyzed congeners.

		LOQ	LOD
PCB Congener	**8**	0.5	0.1
	18	0.5	0.1
	28/31	0.25	0.1
	44	0.1	0.1
	45	0.1	0.1
	47	0.25	0.1
	49	0.5	0.1
	52	0.5	0.1
	56/60	0.5	0.25
	66	0.5	0.1
	70	0.5	0.1
	74	0.1	0.1
	77	0.5	0.25
	81	1	0.25
	87	1	0.25
	95	1	0.25
	105	1	0.1
	110	1	0.25
	114	0.25	0.1
	118	0.5	0.25
	123	0.1	0.1
	128	0.5	0.25
	132/153	0.25	0.1
	146	1	0.1
	149	1	0.1
	151	0.5	0.25
	156	0.5	0.1
	157	1	0.25
	167	1	0.25
	169	1	0.25
	170	2.5	0.1
	174	0.5	0.25
	177	0.5	0.25
	180	2.5	0.25
	183	0.5	0.25
	187	0.5	0.25
	194	1	0.1
	195	1	0.1
	206	1	0.1
	209	1	1

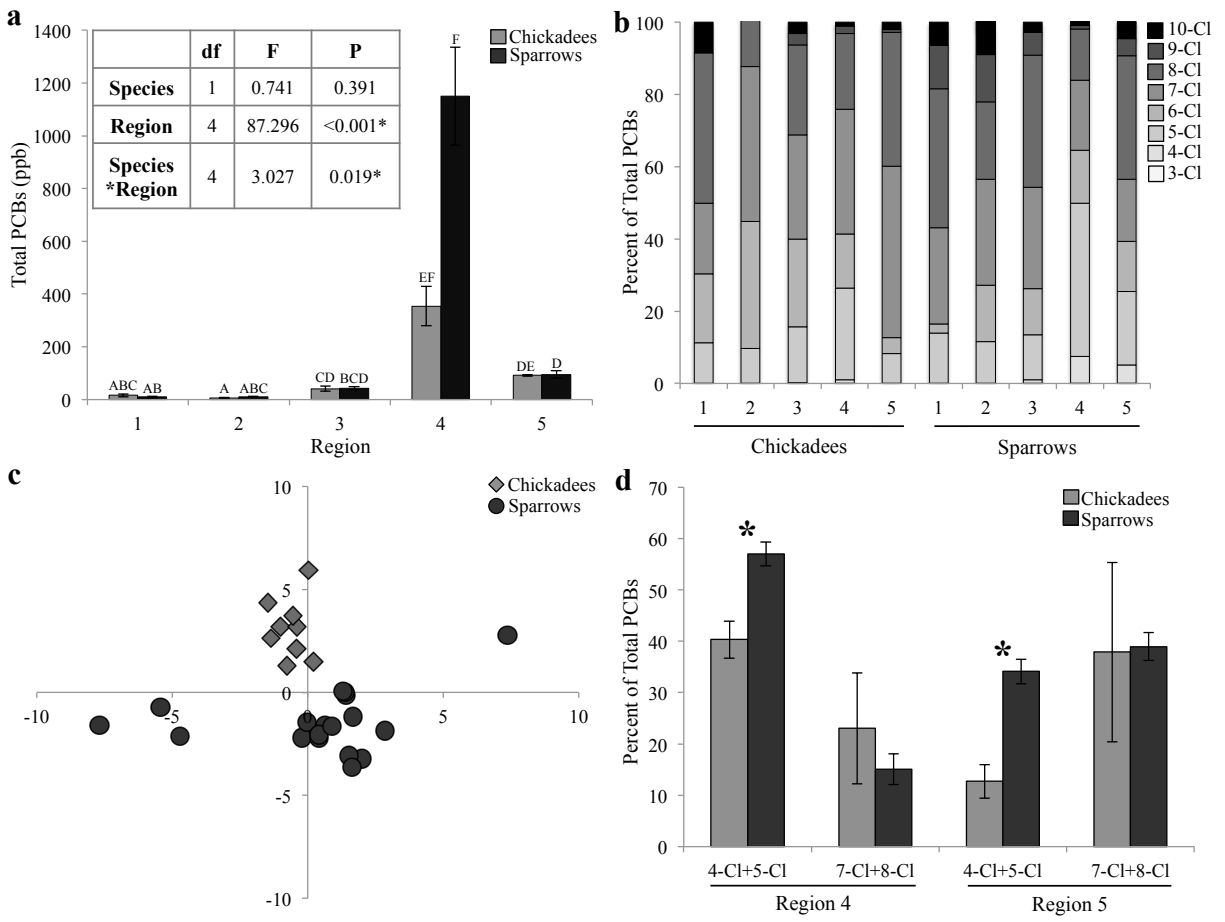

Figure 2.3. Differential bioaccumulation of PCBs in two different songbirds. **a)** Total PCB concentration (ppb, mean±SE) were analyzed in blood samples of black-capped chickadees and song sparrows from different regions in northeastern New York State. ANOVA (inset) was performed on log-transformed data. Non-overlapping letters indicate statistical difference between groups (Tukey's post hoc, $P<0.05$). **b)** The PCB chlorination profiles for black-capped chickadees and song sparrows by region. **c)** Principle component analysis of PCB congener profiles from black-capped chickadees and song sparrows from the region with highest historical PCB contamination (Region 4). PC1 accounts for 21% of the variability, and PC2 accounts for 19.3% of the variability. **d)** The proportion of lower chlorinated (4-Cl+5-Cl) and higher chlorinated (7-Cl+8-Cl) PCBs in black-capped chickadees and song sparrows from Region 4 (Student's *t*-test: t=-3.36, $P=0.0016$) and Region 5 (Student's *t*-test: t=-2.66, $P=0.01$).

Black-capped chickadee song

Male black-capped chickadees are known to have a highly stereotyped song, with the glissando and interval ratios varying less than 2% across their entire North American range (Figure 2.2a) (Christie et al. 2004). Although black-capped chickadees showed glissando and

interval ratio variation for all regions (Figure 2.4a, b), only the glissando ratio in Regions 2-5 showed variation greater than 2%, while the interval ratio variation compared to published values (Christie et al. 2004; Weisman et al. 1990) was less than 2% in all regions (Table 2.4). Region 1 had the lowest glissando ratio, which was significantly different from Regions 4 and 5 (Figure 2.4a; Kruskal-Wallis test: $P<0.05$, Wilcoxon post hoc with Bonferroni correction: $P<0.05$). Region 5 had the highest glissando ratio. The interval ratio values did not vary significantly between regions (Figure 2.4b; ANOVA: $F=1.93$, $P=0.10$).

In addition to black-capped chickadee population differences in glissando ratio in Regions 2-5 (Table 2.4), there is variation in individual black-capped chickadees singing glissando ratios in areas of PCB contamination (see Figure 2.5 for examples). The proportion of individual chickadees singing inconsistent glissando ratios was higher in Regions 4 and 5, than in Regions 1-3 (Figure 2.4c; Chi-square=22.81, df=4, $P=0.0001$). The proportion of individual chickadees singing inconsistent interval ratios was not significantly different between regions (Figure 2.4d; Chi-square=4.68, df=4, $P= 0.32$).

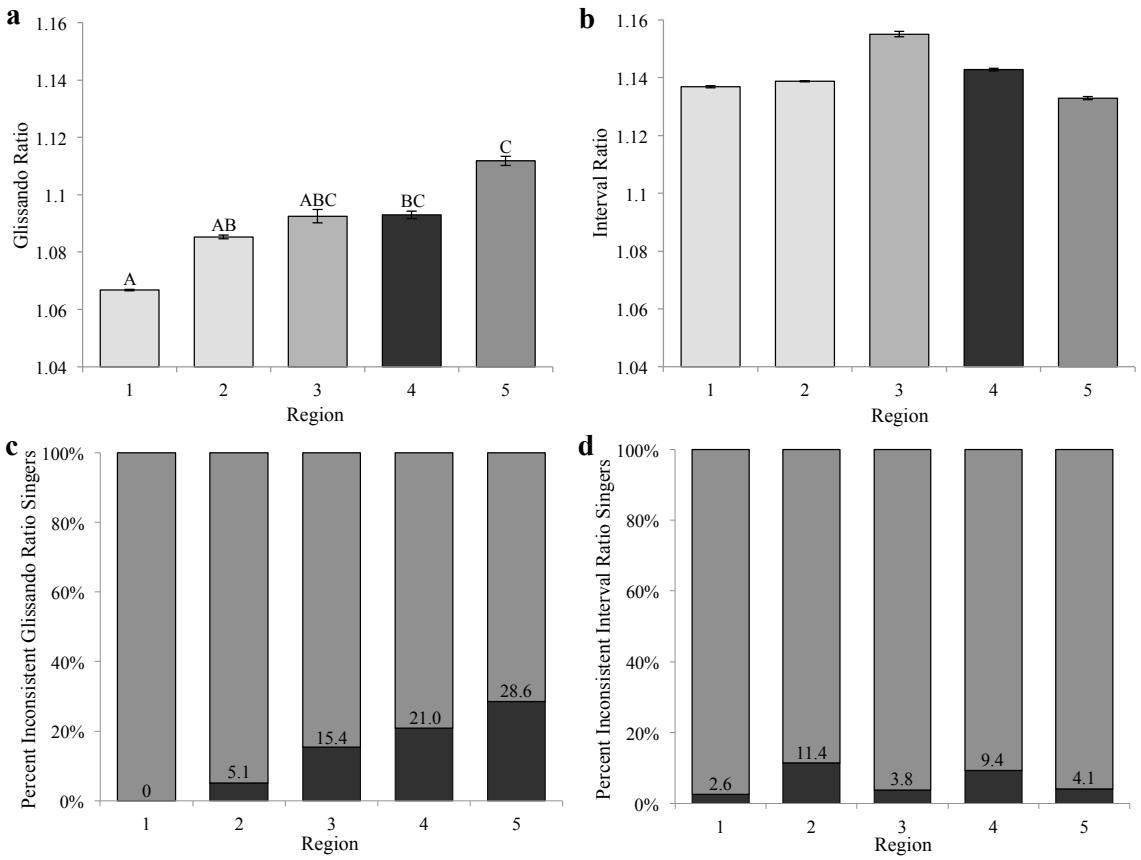

Figure 2.4. Black-capped chickadees have more variable songs in regions of PCB contamination. **a)** Average glissando ratio of black-capped chickadees by region. Bars are mean±SE and non-overlapping letters indicate statistical difference between the regions (Kruskal-Wallis test, $P<0.05$; Wilcoxon post hoc with Bonferroni correction, $P<0.05$). **b)** Average interval ratio of black-capped chickadees by region. Bars are mean±SE (ANOVA, $P>0.05$). **c)** Proportion of consistent and inconsistent glissando ratios of individual black-capped chickadees. Individuals singing consistent glissando ratios (gray, CV<0.03) and inconsistent glissando ratios (black, CV>0.03) are shown by region (Chi-squares=22.81, df=4, $P=0.0001$). The numbers in bars are the actual percentages of individual chickadees singing glissando ratios with a CV>0.03. **d)** Proportion of consistent and inconsistent interval ratios of individual black-capped chickadees. Individuals singing consistent interval ratios (gray, CV<0.03) and inconsistent interval ratios (black, CV>0.03) are shown by region (Chi-squares=4.68, df=4, $P=0.32$). The numbers in bars are the actual percentages of individual chickadees singing interval ratios with a CV>0.03.

Table 2.4. Black-capped chickadee glissando and interval ratios by region. Ratios are shown as mean±SD. Percent deviation is calculated from published ratio values in Weisman *et al.* (Weisman et al. 1990).

Region	Sample Size	Average Glissando Ratio±SD	Average Interval Ratio±SD	%Glissando Deviation	%Interval Deviation
1	38	1.067±0.017	1.137±0.017	1.023	0.253
2	71	1.086±0.027	1.140±0.021	**2.878**	0.517
3	34	1.088±0.035	1.149±0.018	**3.068**	1.315
4	43	1.093±0.035	1.143±0.020	**3.502**	0.777
5	49	1.112±0.049	1.133±0.017	**5.286**	-0.098

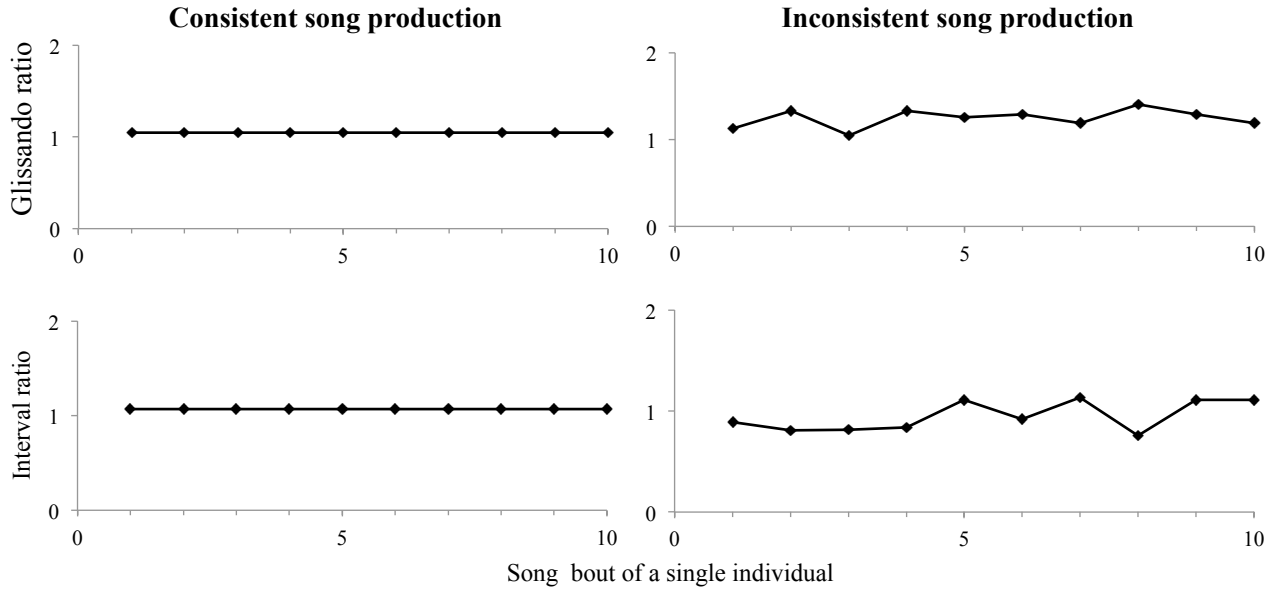

Figure 2.5. Stereotyped and variable glissando and interval ratios of black-capped chickadees.

Song sparrow song

When all trills recorded from male song sparrows during 2006-2007 were plotted as trill rate (Hz) versus bandwidth (kHz), the regression line (Figure 2.6a) was comparable to published values (Podos 1997). The proportion of song sparrows that sing high performance (low deviation) trills was significantly greater (Figure 2.6b; Chi-square=12.80, df=4, *P*=0.01) in Region 4 than other regions along the Hudson River. The 2009 subset of song sparrows that were sampled for

blood and recorded show that as total PCB concentration in the blood increases the trill rate decreases (Figure 2.6c; ANOVA: F=5.08, *P*=0.03).

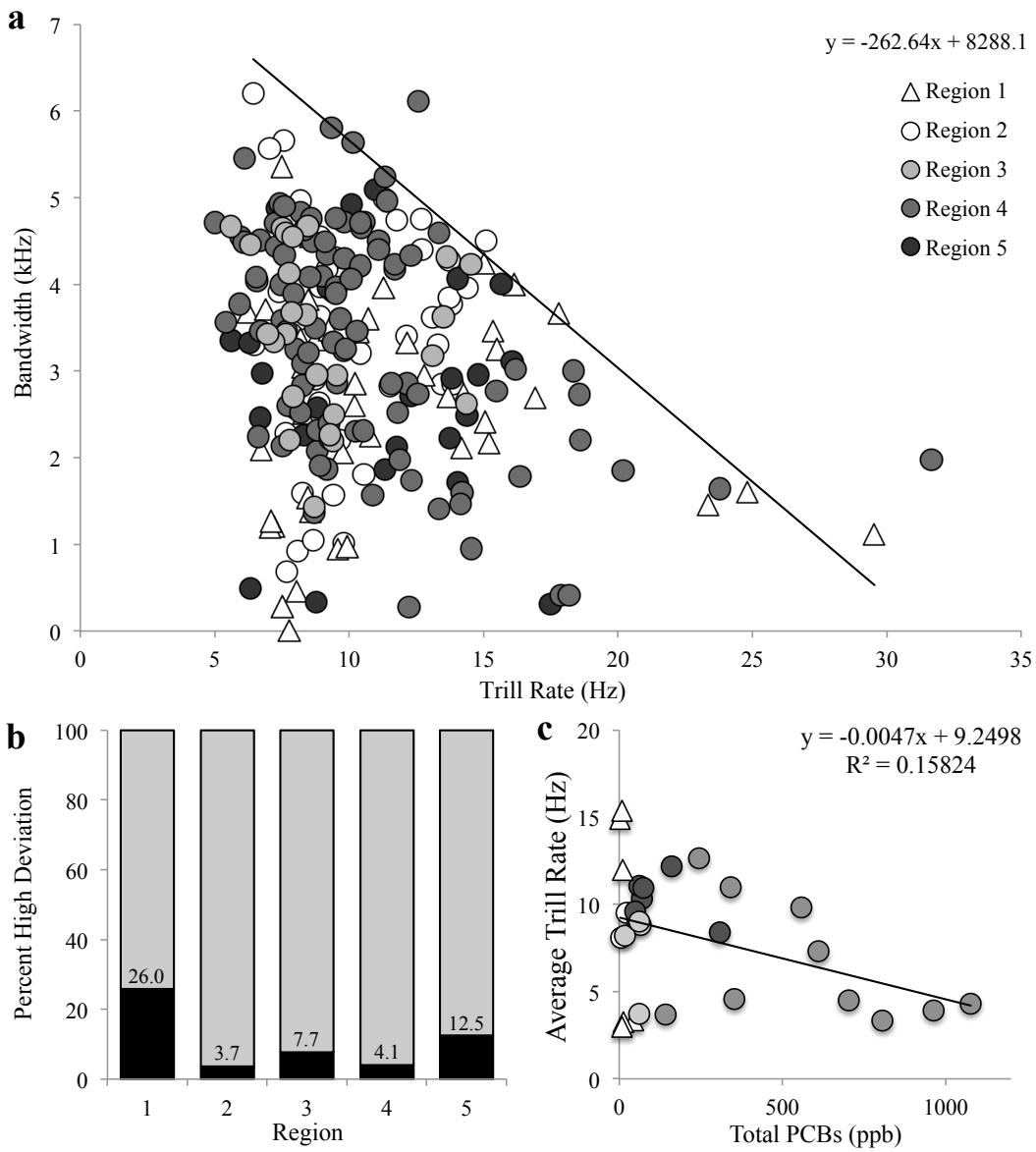

Figure 2.6. Song sparrows have higher performance trills in regions of PCB contamination. **a)** Trill rate-bandwidth tradeoff of song sparrows. The regression line is calculated from all recorded trills, and each point represents a randomly chosen trill type of one individual song sparrow. **b)** Proportion of low and high deviation song sparrow trills. Individuals singing low deviation (high performance, gray, distance<4) or high deviation (low performance, black, distance>4) trills by region (Chi-square=12.80, df=4, *P*=0.01). The numbers in bars are the actual percentages of individual sparrows singing high deviation (low performance) trills with a distance from the regression line >4. **c)** Total PCBs (ppb) and trill rate (Hz) correlation in 2009 song sparrows (ANOVA: F=5.08, *P*=0.03).

Body condition and mercury concentration

Black-capped chickadees captured from the five regions showed no differences in wing chord length, body weight, body condition index, fat score, or feather ectoparasite damage (ANOVA's: $P>0.05$), but differed in tarsus length (ANOVA: F=3.75, $P=0.01$), with Region 5 birds having significantly longer tarsi than Regions 2 and 3 (Tukey's test: $P<0.05$). Song sparrows captured from the five regions showed no differences in wing chord length, tarsus length, body weight, or feather ectoparasite damage (ANOVA's: $P>0.05$), but differed in body condition index (ANOVA: F=3.63, $P=0.0079$), with Region 4 birds having significantly higher body condition indices than Region 5 (Tukey's post hoc test: $P<0.05$). Sparrows also differed in their fat score (ANOVA: F=5.29, $P=0.0006$), with Region 5 birds having higher fat scores than Regions 2 and 4 (Tukey's post hoc test: $P<0.05$). Song sparrows from Region 2 had higher blood mercury concentrations than song sparrows from the other regions (Figure 2.7; ANOVA: F=6.58, $P=0.0025$).

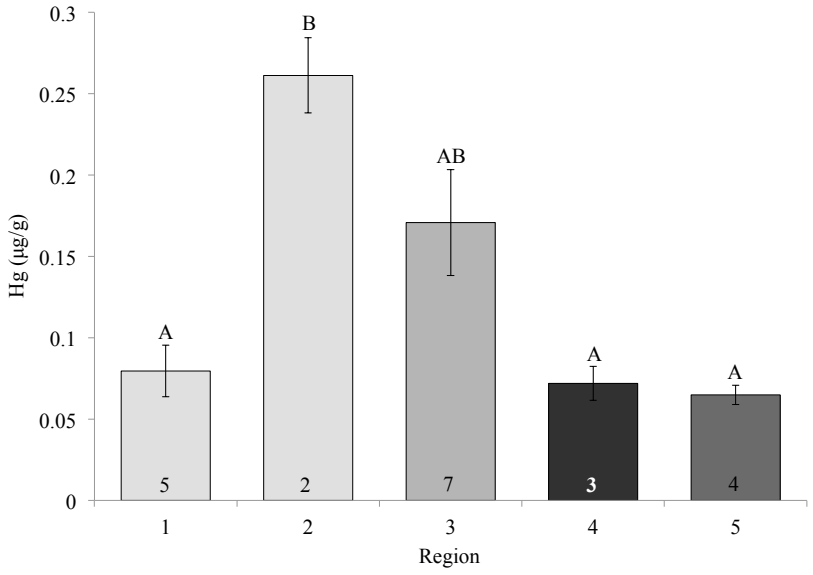

Figure 2.7. Mercury concentrations in song sparrow blood from 2008. Bars are mean±SE and non-overlapping letters indicate statistical difference between the regions (ANOVA: F=6.58, $P=0.0025$).

Discussion

The results of this field study suggest that the historical contamination of the Hudson River is reaching the terrestrial breeding bird populations of black-capped chickadees and song sparrows, and influencing male song production. Both black-capped chickadees and song sparrows have elevated total PCB concentrations in Region 4 (Figure 2.3a). Yet, likely due to differences in life history of the two species, the PCB congener composition of the black-capped chickadees and song sparrows differ significantly (Figure 2.3c, d). Although both species predominantly eat insects during the breeding season (Bent 1964; Judd 1899), song sparrows are a riparian species (Stauffer & Best 1980) and therefore usually have territories and nesting sites closer to the contaminated Hudson River than the black-capped chickadees, which commonly inhabit nearby woodlands (Foote 2010). Accordingly, song sparrow blood had a greater concentration of total PCBs, and their PCB profile more closely reflected the congener profile found on the Hudson River (EPA 1997; Trustees 2005), while the chickadee PCB profile indicated a lower level of exposure (total PCBs), as well as a congener profile that was more evenly distributed.

In black-capped chickadee song, the glissando ratio is thought to be important in signaling species identity (Weisman et al. 1990). Therefore, the greater than 2% glissando ratio variation in Regions 2-5 (Table 2.4) is likely indicative of a biologically significant deviation of the species identity signal (Christie et al. 2004). Individual males that reproduce consistent glissando and interval ratios within bouts of singing are dominant, high quality males, that reliably broadcast their species identity (Christie et al. 2004; Weisman et al. 1990). While some variation in the proportions of consistent and inconsistent individual black-capped chickadee singers might be expected within each region, our results indicate a higher proportion of inconsistent individual

glissando ratio singers in the PCB contaminated regions (4 and 5), compared to Regions 1-3 (Figure 2.4c). Because the glissando ratio has been identified as being important in species recognition (Christie et al. 2004), this result has critical implications for chickadee populations in these areas. If PCB levels are affecting the ability of black-capped chickadees to reproduce consistent glissando ratios, this may compromise species recognition and influence population dynamics (Grant & Grant 1996; Weisman et al. 1990).

Similar to the chickadees, song sparrows also showed altered song characteristics along the Hudson River. Conversely, the song sparrows had a significantly larger proportion of higher performance trills in areas with PCBs (Figure 2.6b). This pattern in the trill rate-bandwidth tradeoff may be driven by the bandwidth, since the trill rate decreased in song sparrow individuals with higher blood PCB concentrations (Figure 2.6c). Considering that much of song sparrow song variability is embedded in trill type and note complexity, this relationship between PCB blood concentrations and trill performance is a significant finding (Markman et al. 2008). The trill rate-bandwidth tradeoff is a conservative measure of song quality in song sparrows, therefore it is likely that even more pronounced differences exist in other aspects of song.

Male song quality and body condition covary in many species (Galeotti et al. 1997). However, with both the black-capped chickadees and the song sparrows, the differences in body condition variables among regions were inconsistent with the alternative hypothesis that condition explained song variation.

Additionally, heavy metals have been previously shown to effect bird song characteristics (Gorissen et al. 2005), and mercury is a historic pollutant in the area (Lorey & Driscoll 1999). Therefore, although the PCB gradient along the Hudson River does correlate with song variation in black-capped chickadees and song sparrows, and it is known that the passerine song system is

sensitive to PCBs (Hoogesteijn et al. 2008), it is important to consider that PCBs may not be the only pollutant in the region affecting birdsong. Mercury concentration in song sparrow blood was elevated in individuals from Region 2, however this result does not adequately explain the observed song variation. Mercury is a neurotoxin (Wolfe et al. 1998), therefore, in Region 2 we would expect lower song quality for both species. Instead we actually see higher quality song in black-capped chickadees and song sparrows in Region 2. Therefore, the patterns in chickadee and song sparrow songs are not explained by mercury exposure.

Results from this study indicate that black-capped chickadees are singing lower quality songs in areas with high PCBs in the environment, and song sparrows are singing higher quality songs in areas with high PCB contamination. This dichotomy in song results is likely linked to the high PCB blood levels in contaminated PCB regions, and the different congener profiles found between the two species. The higher chlorinated PCBs that are found predominantly in black-capped chickadees may have toxic effects, resulting in the inability of a higher proportion of individuals living in PCB contaminated regions to sing stereotyped glissando ratios. The lower chlorinated PCBs that are found predominantly in the song sparrows may be acting as hormone mimics, resulting in a greater proportion of high performance trills in PCB contaminated regions. Indeed, hormone mimics have been shown previously to increase European starling (*Sturnus vulgaris*) song complexity and increase HVC (Markman et al. 2008), a result that may be occurring in song sparrows as well.

Although the critical consequences of PCB-exposure have been broadly explored, the effects of lower PCB levels are not yet resolved. To our knowledge, this is the first study to investigate the effect of sublethal PCB levels on birdsong. Our results show that non-destructive sampling methods are successful in quantifying congener specific PCB levels in the blood of wild

passerines. Furthermore, we show that the behavioral endpoint of birdsong is altered in areas of PCB contamination, suggesting that sublethal levels of PCBs are impacting wildlife communication.

PCBs have been implicated in behavioral changes in human populations (Huisman et al. 1995; Lundqvist et al. 2006)). Therefore, in conjunction with our results, the available data strongly advocates that further research investigating the behavioral effects of sublethal PCBs across all taxa are vital to fully understanding this harmful worldwide chemical pollutant. We specifically suggest expanding field studies to understand whether the pattern of high- and low-PCB chlorination is affecting other species similarly. In addition, laboratory research is needed to establish a causal relationship between sublethal dietary PCB levels and behavioral changes, including song.

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Chapter 3:

POLYCHLORINATED BIPHENYLS ALTER MALE ZEBRA FINCH SONG NUCLEI AND SONG CHARACTERISTICS

Abstract

Polychlorinated biphenyls (PCBs) are chemical pollutants that are found worldwide and are present in organisms at virtually every trophic level. Although the acute effects of PCBs are well documented, research on the sublethal consequences of PCB-exposure on animal behavior is limited. Song is a vital aspect of bird communication and subsequent reproductive success, and previous studies have shown that sublethal PCB-exposure affects the song system in the developing avian brain. This experiment investigates the effects of Aroclor 1242- and PCB 52-exposure during early development on adult song characteristics and neuronal morphology in song nuclei in male zebra finches (*Taeniopygia guttata*). Results show that song content for PCB-treated males is similar to that of control males, but the treated males reproduce their song with less variability. In addition, PCB-exposure resulted in an increase in dendritic spines in the song motor area HVC. PCB-exposure did not affect the number of dendritic spines in the RA. Treatment was associated with a tendency toward fewer dendritic spines in the hippocampus. This experiment adds new insights to the impact of sublethal levels of PCBs on developing birds, and highlights the necessity of additional research to better understand the effects of contaminated environments on wild bird populations.

Introduction

Polychlorinated biphenyls (PCBs) are synthetic chemical pollutants that are ubiquitous in the environment and present in organisms at virtually every trophic level and on every continent (Brinkman & de Kok 1980; Clarkson 1995; Safe & Hutzinger 1987; Waid 1986). High levels of PCB exposure can cause acute effects such as cancer and mortality ((Bleavins et al. 1980; Pavuk et al. 2004), and an increasing number of studies show that sublethal exposure also can have diverse detrimental effects (e.g. Helberg et al. (2005); Lundqvist et al. (2006)). PCBs are environmental endocrine disruptors (Tyler et al. 1998). Over 200 PCB congeners have been produced, by varying the di-benzene backbone structure and the degree of chlorination, and these can act in concert on organisms in the wild (e.g. Echols et al. (2004)). Due to the chemical structure of PCBs, one sublethal consequence should be alterations to animal behaviors, especially behaviors that are linked to circulating steriods, however only a limited number of studies have investigated this (but see (Hoogesteijn et al. 2005; McCarty & Secord 1999a)).

Song is a vital aspect of bird communication and subsequent reproductive success (Kroodsma 1996). In most songbird species, song is learned during an early period of the bird's development and is highly stable when produced in adulthood. One of the most basic functions of song is that it signals species identity. Many female passerine species also use male song in selecting a mate, presumably because it is an honest indicator of quality (Searcy 1996). For example, male repertoire size has been shown to predict initial mating success and is correlated to fitness in male song sparrows (*Melospiza melodia*) (Reid et al. 2004, 2005). In addition, great tits (*Parus major*) that sing 'better' have higher individual lifetime reproductive success (Lambrechts & Dhondt 1986), while female canaries (*Serinus canaria*) prefer males that sing more complex songs (Spencer et al. 2005a). Males of many species also use song to define territories (Krebs et

al. 1978). Thus, large changes in song characteristics could prevent conspecific recognition, and even small changes could affect the establishment and defense of territories, mate attraction, pair bond maintenance, and ultimately, reproductive success.

In oscine passerines song is learned and produced by a set of brain nuclei that develop and function under the control of the endocrine system (Ball et al. 2002). The song production pathway consists of the HVC (involved in organizing the song (both the syllables and their order) as a whole) that projects to the robust nucleus of the arcopallium (RA; involved in production of individual notes) that projects directly and indirectly (via the dorsomedial nucleus of the intercollicular complex) to the hypoglossal (nXII) nucleus, that in turn controls the muscles of the syrinx used for vocalizations (DeVoogd 2004). Each of these brain areas has high levels of androgen and/or estrogen receptors (DeVoogd & Lauay 2001), and the close integration of the endocrine and neural systems suggests that any agent disrupting these endocrine receptors is likely to disrupt the behaviors organized by these regions. Experimentally altering steroid level in juvenile birds prevents full development of these brain nuclei and of song (DeVoogd & Lauay 2001). Additionally stressors, especially early in development, impair song (Laiolo & Tella 2005; Spencer et al. 2005a; Spencer et al. 2005b) and prevent full development in the brain areas responsible for song (Buchanan et al. 2004; Nowicki et al. 2002). Therefore, any agent interfering with brain or endocrine physiology is likely to affect the organization of these brain areas and may result in the impairment of song acquisition or production (for examples see Gorissen et al. (2005); Iwaniuk et al. (2006); Markman et al. (2008)). In particular, PCBs are likely to cause such neural behavioral effects because of their actions on steroid receptors and receptor function (Bonefeld-Jorgensen et al. 2001).

Previous studies show that exposure to sublethal levels of PCBs in young birds should impair adult song production through effects on the developing brain (Hoogesteijn et al. 2008). When song nuclei volumes were measured in the progeny of Aroclor 1248-treated zebra finch (*Taeniopygia guttata*) mothers, the RA was significantly smaller in both male and female offspring (Hoogesteijn et al. 2008). Yet, currently it is not known whether these effects on the developing brain resulted in behavioral consequences, although results from the field indicate that aspects of song in wild populations differ between PCB-contaminated sites and sites that are not contaminated (citation of Thesis: Chapter 2). Therefore, our study provides the necessary link between the current experimental knowledge of the effects of PCBs on the avian song system and correlations between PCB load and song variation in wild birds.

In this experiment we treated male zebra finch nestlings with environmentally relevant doses of Aroclor 1242 and PCB 52 and measured song characteristics and dendritic spines in the adult birds. Aroclor 1242 is a commercial PCB mixture that has approximately 42% by mass chlorine composition. It was the predominant industrial mixture released on the Hudson River by General Electric in northeastern New York State last century (EPA 1997), making the results from this experiment maximally comparable to findings from Hudson River field sites (citation of Thesis: Chapter 2; (McCarty & Secord 1999b, 2000)). We predicted that male zebra finch exposed to the Aroclor 1242 mixture as nestlings would exhibit decreased song learning capability, decreased song performance capability, and fewer dendritic spines in the song nuclei. Because early developmental exposure to Aroclor 1248 decreased RA volume in zebra finches (Hoogesteijn et al. 2008), we expect detrimental results on song learning, song production, and the song system with exposure to Aroclor 1242, a commercial mixture with similar chlorine content.

PCB 52 is a tetra-chlorinated PCB congener with chlorines at the 2, 2', 5, 5' positions around the di-benzene backbone. PCB 52 is known to have estrogenic properties (Jansen et al. 1993), and the chemical structure has estrogen receptor affinity (deCastro et al. 2006). In contrast to the Aroclor 1242 treatment, we predict that nestlings exposed to PCB 52 would exhibit increased song learning capability, increased song performance capability, and more dendritic spines in the song nuclei. Because PCB 52 is a known estrogenic congener (Jansen et al. 1993), we expect song learning, song production, and dendritic spines all to increase due to exposure early in development to an estrogen mimic (Adkins-Regan et al. 1994). This experiment attempts to add to our knowledge about the effect of PCBs on birdsong, and to specifically examine whether developmental PCB-exposure affects male song characteristics and song nuclei neuronal morphology.

Methods

Experimental subjects

All zebra finches used in this study were from the colony of T. DeVoogd and housed in Uris Hall, Cornell University (Ithaca, New York, USA). The Cornell University Institutional Animal Care and Use Committee approved all animal procedures (IACUC Protocol 1988-0135). Zebra finches were fed with Kaytee™ forti-finch diet, oyster shell (a grit and calcium source), cuttlebone (for calcium and beak maintenance), and water *ad libitum*. Individuals were banded as nestlings with a unique color band combination for identification (Avinet, Inc. Dryden, New York 13053-1103 USA). Day 1 for each chick was defined as the day it hatched. Subjects were housed in rooms on a 14L: 10D photoperiod, with the humidity maintained between 40-50%.

Thirteen zebra finch pairs with successful breeding experience prior to the start of this experiment were housed in cages (60.96cm x 35.56cm x 40.64cm) equipped with one plastic nest cavity and coconut fiber nesting material. Nest boxes were checked daily to monitor nest building, incubation, and laying. Hatching order was recorded by marking each nestling daily with colored Crayola™ non-toxic permanent markers until day 14 when they were uniquely color banded for identification. The male nestlings of these nests were the subjects of this experiment and were randomly assigned to one of three treatment groups.

Genetic sexing

The sex of each nestling was determined genetically and confirmed with adult plumage by day 60. One pinfeather, or 3-5 down feathers was used as genetic material. DNA was extracted using Qiagen DNeasy™ Blood & Tissue protocol (QIAGEN, Catalogue number 69504, Valencia, California, USA). The feather quill was submerged in buffer solution and lysed for 1-3 hours at 56ºC prior to extraction. The final elution volume was between 50-75µL. *Taq* PCR Kit (New England BioLabs® Inc., Catalogue number E5000S, Ipswich, Massachusetts, USA) was used for PCR amplification. Each 9.6µL PCR contained 2µL of template DNA, 5.9µL ddH_2O, 1µL 10x buffer, 0.4µL 50mM $MgCl_2$, 0.2µL 10mM dNTPs, 0.1µL Platinum *Taq*, and 0.2µL of each of the two 10µM W-allele primers (Kahn & Quinn 1998). The following PCR program was used for DNA amplification: an initial denaturation step of 90ºC for 2 minutes; followed by a 7-cycle touchdown: 94ºC for 50 seconds, 57-51ºC for 45 seconds (dropping 1ºC per cycle), 72ºC for 1 minute; another 30 cycles of 94ºC for 50 seconds, 50ºC for 45 seconds, then 72ºC for 1 minute; and a final hold temperature of 4ºC. Genetic procedures were performed in the Evolutionary Genetics Core Facility at Cornell University (Ithaca, New York 14853 USA).

Experimental treatment

The dosage for each group was based on the only other known study of PCB dosage to zebra finches nestlings (Hoogesteijn 2003) and modified to more closely match what nestlings may encounter in areas of environmental PCB contamination (Echols et al. 2004; Maul et al. 2006; McCarty 2002; Secord et al. 1999). The solutions were administered to the nestlings from day 2 until day 8 using a 25µL blunt-tipped Hamilton™ syringe. The solutions were administered by taking advantage of the gaping reflex: the nestling gape reflex was stimulated with a slight touch and the solutions were drop-fed into the gape. To prevent cross-contamination, each treatment had a dedicated syringe that was flushed with hexane and wiped clean between each use. Fledglings remained in their natal cage until post-hatch day 50, and on post-hatch day 51 they were placed in single-sex aviaries (91.44cm x 60.96cm x 121.92cm) in a separate room from their parents.

Canola oil (control) treatment

Male nestlings assigned to the control group (N=17) were administered a total of 165µL of Canola oil across seven days. The nestlings were dosed with 10µL of Canola oil on day 2, 15µL on day 3, 20µL on day 4, and the 30µL on days 5 through 8.

Aroclor 1242 treatment

Male nestlings assigned to the Aroclor 1242 group (N=7) were administered a total of 165µL of Aroclor 1242 (AccuStandard.com) dissolved at 1-mg/mL in Canola oil across seven days. The nestlings were dosed with 10µL of the Aroclor 1242 solution on day 2, 15µL on day 3, 20µL on day 4, and the 30µL on days 5 through 8.

PCB 52 treatment

Male nestlings assigned to the PCB 52 group (N=7) were administered 165µL of PCB 52 (AccuStandard.com) dissolved at a 1-mg/mL concentration in Canola oil across seven days. The nestlings were dosed with 10µL of the PCB 52 solution on day 2, 15µL on day 3, 20µL on day 4, and the 30µL on days 5 through 8.

Song recording

The experimental males were recorded on day 120 (±1 day). Each recording session lasted for one hour. The same experienced, unrelated, adult female was used as a stimulus in all song recordings. Songs were recorded with a Tascam HD-P2 Recorder, a Universal Telinga Pro 24-inch Parabola, and a Sennheiser ME62 Omni Microphone. Recordings were made directly onto a 4GB flash card in a sound proof room. The recordings from all males were analyzed with Sound Analysis Pro (SAP; http://ofer.sci.ccny.cuny.edu/sound_analysis_pro) at 44.1kHz and 16-bits. SAP is an open access song analysis program designed to specifically analyze zebra finch songs (Tchernichovski 2004). The individual motif (song type) of each male was identified as the smallest repeated group of notes. Between 8 and 10 motifs were clipped out of each hour-long recording for the SAP analysis. The set of motifs for each experimental male was compared with his father's set of motifs to determine motif similarity between father and sons. The set of motifs for each experimental male was also compared with itself to compare consistency of the motif within a male. The percent similarity (assessment of the liklihood that the two sounds are related to eachother), the percent accuracy (quantification of the accurracy of the vocal match between the two sounds being compared), and the percent sequential (accounting for the temporal order, or syntax, of the sounds when scoring similarity) were measured with SAP in each comparison (see Figure 3.1 for spectrogram examples of measurements). The average percentage score was taken

for each comparison for each individual treatment male. Therefore, each male has six scores: three (percent similarity, accuracy, and sequential) scores from his comparison with his father, and three (percent similarity, accuracy, and sequential) scores from his comparison with himself.

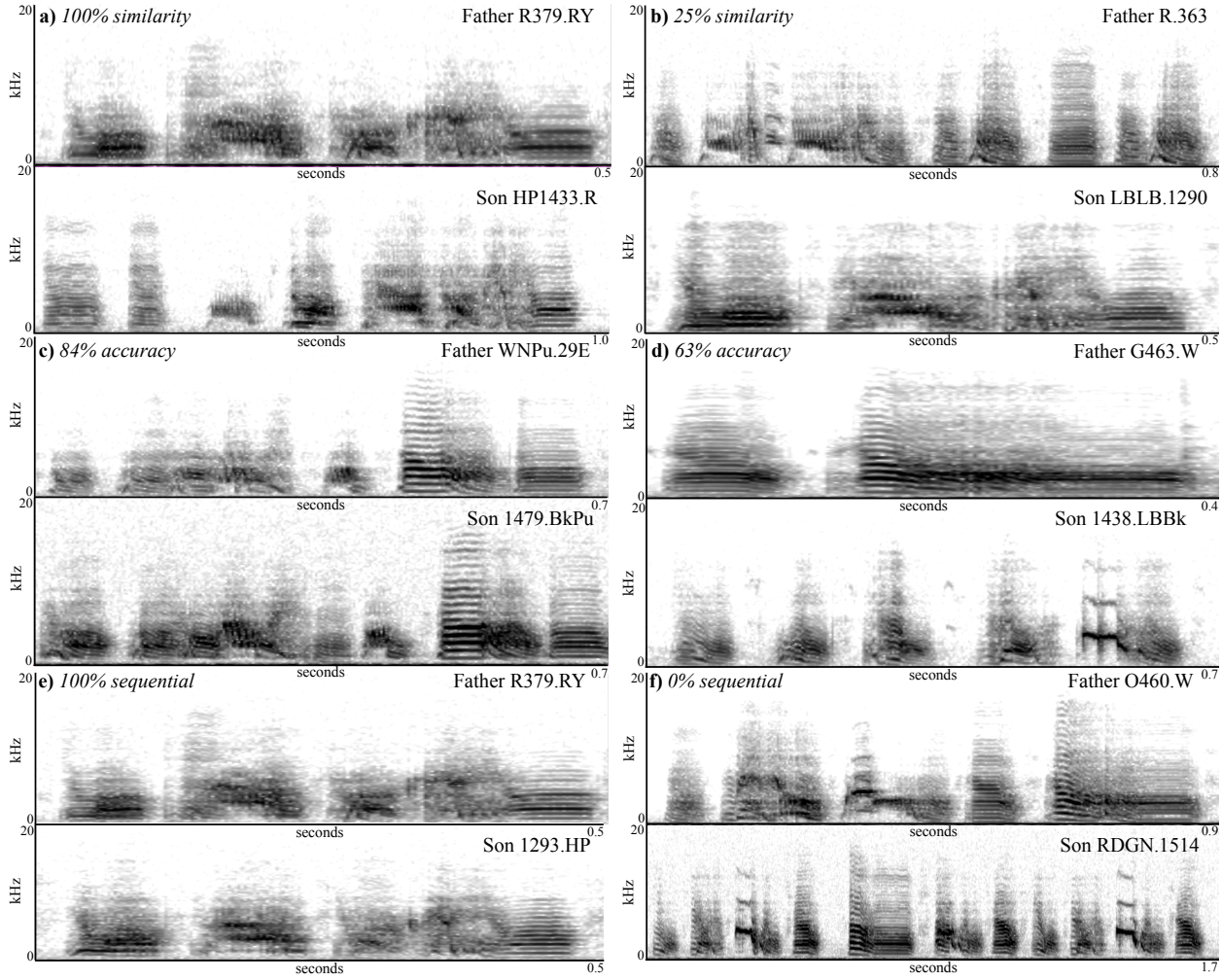

Figure 3.1. Spectrograms of represnetative male zebra finch motifs. Each spectrogram father (above)/son (below) pair has either the highest average overlap score (similarity **(a)**, accuracy **(c)**, sequential **(e)**) or the lowest average overlap score (similarity **(b)**, accuracy **(d)**, sequential **(f)**) of all pairwise comparisons.

Histological preparation

Between approximately 200-300 days of age, the experimental males were anesthetized with 0.1 mL Chloropent and were transcardially perfused with 0.9% saline followed by 10% formalin. Immediately after extraction, the fresh brain was weighed and then immersed in Golgi-

Cox solution (modified from Glaser and Van der Loos (1981)) for approximately 6 weeks, with the solution changed after one week, when the brain was weighed again. The tissue was dehydrated and embedded in celloiden, sectioned at 100µm in the coronal plane, reacted with ammonia, counter-stained with methylene blue and cresyl violet, and mounted on slides with coverslips. After the perfusion was completed, the testes were also removed and weighed.

Brain quantification

Neurons were selected for quantification in the song nuclei areas of the HVC and the RA, and in the hippocampus, an area important in spatial memory that was used to control for random variation in stain quality. Within each of the three brain sites, spine density was counted using a 945x magnification with oil for three 11-12µm dendrite samples that were parallel to the plane of the section. Dendritic spines were counted on a proximal location (approximately 11-12µm from the soma), a medial location (approximately 22-24µm from the soma), and a distal location (the last 11-12µm, usually approximately 40µm from the soma). When possible, all three locations were measured from the same dendrite. When it was not possible to get all measurements from the same dendrite, additional measurements were taken from other dendrites to increase the sample number. We measured HVC and RA spine density along different regions of the dendrite because it is know to be dependent on the developmental environment (Hill & DeVoogd 1991; Lauay et al. 2005; Nixdorf-Bergweiler et al. 1995). Therefore, we expected this detailed measurement of neuronal morphology to compliment and extend previous work on the effect of PCBs on volume of song nuclei (Hoogesteijn et al. 2008).

In each experimental male, the dendritic spines from the proximal, medial, and distal regions, on three dendrites, from each of the three regions were counted, for a maximum of 27 counts from each individual. All spines were counted, included those that looked like a swelling

of the dendritic surface, and spines that were long, thin projections. Due to perfusion complications, uneven staining, and poor mounting, we were prevented from obtaining a full count sample from each experimental male used in the song analysis. The resulting sample size for the dendritic quantification was N=11 for the HVC, N=14 for the RA, and N=16 for the hippocampus. The experimenters were blind to the treatment of the individual throughout the quantification.

Statistical analysis

Each data set was checked for normality, and then analyzed with a mixed model, where treatment was a fixed effect. Data are reported as mean±SE and significance is noted at the $P<0.05$ (*) and $P<0.10$ (†) levels.

Results

There were no differences between the mass of the total brain weighed immediately after perfusion, or after the brain was immersed in Golgi-cox solution for one week (Figure 3.2; ANOVA: Brain Mass-1: $F_{2,26}=0.80$, $P=0.46$; Brain Mass-2: $F_{2,26}=0.95$, $P=0.40$). There were two testes from each male, and the males treated with Aroclor 1242 had increased testes weight at marginal significance at the time of perfusion (Figure 3.2; ANOVA: $F_{2,26}=3.25$, $P=0.06$).

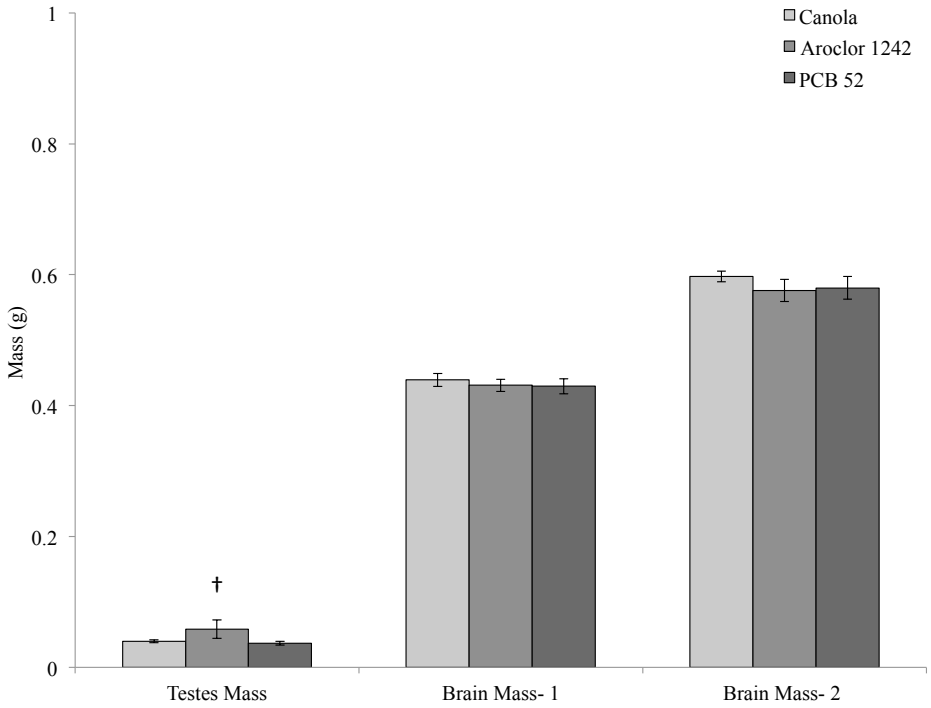

Figure 3.2. Testes and total brain mass. Average testes mass and brain mass-1 measured immediately after perfusion (±SE). Brain mass-2 measured after the brain was immersed in Golgi-cox solution for one week (±SE). Differences among treatment groups were analyzed with an ANOVA (Testes Mass: $F_{2,26}$=3.25, *P*=0.06; Brain Mass-1: $F_{2,26}$=0.80, *P*=0.46; Brain Mass-2: $F_{2,26}$=0.95, *P*=0.40).

Song comparisons

When the songs of the treated males were compared to the songs of their fathers, there were no differences in the percentage of overlap in similarity, accuracy, or sequence among the three treatment groups (Table 3.1; ANOVA: Similarity: $F_{2,28}$=0.13, *P*=0.88; Accuracy: $F_{2,28}$=0.15, *P*=0.86; Sequential: $F_{2,28}$=1.46, *P*=0.25). There was no change in the explanation of variance when family was added to the model as a fixed effect.

Table 3.1. Results of song analysis and brain quantification. **a)** Similarity, accuracy, and sequential percent overlap between fathers songs and their sons songs from the three treatment groups (±SE) (ANOVA: Similarity: $F_{2,28}$=0.13, *P*=0.88; Accuracy: $F_{2,28}$=0.15, *P*=0.86; Sequential: $F_{2,28}$=1.46, *P*=0.25). **b)** Similarity, accuracy, and sequential percent overlap within individual sons songs from the three treatment groups (±SE) (ANOVA: Similarity: $F_{2,28}$=2.70, *P*=0.08; Accuracy: $F_{2,28}$=2.87, *P*=0.07; Sequential: $F_{2,28}$=0.12, *P*=0.89). **c)** Average differences among the number of hippocampal spines in the proximal, medial, and distal portion of the dendrite by treatment (±SE) (ANOVA: Proximal: $F_{2,13}$=5.35, *P*=0.008; Medial: $F_{2,13}$=3.60, *P*=0.03; Distal: $F_{2,13}$=2.36, *P*=0.12). **d)** Average differences among the number of HVC spines in the proximal, medial, and distal portion of the dendrite by treatment (±SE) (ANOVA: Proximal: $F_{2,9}$=1.45, *P*=0.23; Medial: $F_{2,8}$=3.50, *P*=0.04; Distal: $F_{2,8}$=0.64, *P*=0.53). **e)** Average differences among the number of RA spines in the proximal, medial, and distal portion of the dendrite by treatment (±SE) (ANOVA: Proximal: $F_{2,11}$=0.08, *P*=0.93; Medial: $F_{2,9}$=0.36, *P*=0.70; Distal: $F_{2,6}$=1.26, *P*=0.30).

		Canola oil	Aroclor 1242	PCB 52
a) Song Comparison with Father	**Similarity**	75.82 (±3.57)	72.42 (±4.32)	70.26 (±8.17)
	Accuracy	73.64 (±0.81)	72.77 (±1.03)	73.07 (±1.21)
	Sequential	62.07 (±4.14)	59.11 (±5.58)	72.43 (±7.9)
b) Song Comparison within Male	**Similarity**	**81.61 (±2.73)†**	**90.82 (±1.82)†**	**88.09 (±3.18)†**
	Accuracy	**81.83 (±1.84)†**	**88.22 (±1.23)†**	**86.53 (±2.28)†**
	Sequential	95.03 (±0.74)	95.46 (±0.71)	95.70 (±0.74)
c) Hippocampus	**Proximal**	**12.80 (±0.62)***	**10.24 (±0.53)***	**12.67 (±0.83)***
	Medial	**13.07 (±0.71)***	**11.14 (±0.51)***	**13.60 (±0.96)***
	Distal	12.27 (±0.64)	10.48 (±0.60)	10.27 (±0.83)
	Total	38.13 (±2.32)	32.50 (±1.48)	36.53 (±1.25)
d) HVC	**Proximal**	**8.78 (±0.70)***	**11.08 (±1.21)***	**9.22 (±0.89)***
	Medial	10.11 (±1.06)	11.85 (±0.89)	8.33 (±0.91)
	Distal	8.89 (±1.03)	10.46 (±1.05)	10.11 (±0.82)
	Total	27.78 (±2.34)	33.38 (±2.80)	27.67 (±2.01)
e) RA	**Proximal**	10.54 (±1.99)	10.13 (±2.88)	10.50 (±2.90)
	Medial	12.00 (±0.61)	11.13 (±1.04)	10.17 (±1.06)
	Distal	11.46 (±1.25)	12.44 (±1.26)	9.83 (±1.05)
	Total	34.00 (±0.71)	33.69 (±1.09)	30.50 (±1.58)

When the songs of the treated males were compared within each male, for a measure of consistency, the percent overlap in similarity and accuracy was different with marginal significance among the treatment groups (Table 3.1; ANOVA: Similarity: $F_{2,28}$=2.70, *P*=0.08; Accuracy: $F_{2,28}$=2.87, *P*=0.07), and there was no difference in the percent of sequential overlap

(Table 3.1; ANOVA: Sequential: $F_{2,28}$=0.12, *P*=0.89). Both Aroclor 1242 and PCB 52 treated males repeated their own song with significantly less variability than males treated with Canola oil (Table 3.1).

Brain quantification

In the hippocampus, the number of spines in the proximal and medial portion of the dendrite differed significantly among treatments (Table 3.1; ANOVA: Proximal: $F_{2,13}$=5.35, *P*=0.008; Medial: $F_{2,13}$=3.60, *P*=0.03). There was no difference among treatments in the number of spines in the distal portion of the dendrite in the hippocampus (Table 3.1; ANOVA: Distal: $F_{2,13}$=2.36, *P*=0.12). Males treated with Aroclor 1242 had approximately 20% fewer spines on their dendrites in the hippocampus than males treated with Canola oil (Table 3.1). Males treated with PCB 52 had fewer spines in the proximal and distal portion of the dendrite, but more spines in the medial portion (Table 3.1).

In the HVC, the number of spines in the medial portion of the dendrite differed significantly among treatments (Table 3.1; ANOVA: Medial: $F_{2,8}$=3.50, *P*=0.04). There was no difference among treatments in the number of spines in the proximal and distal portions of the dendrite in the HVC (Table 3.1; ANOVA: Proximal: $F_{2,9}$=1.45, *P*=0.23; Distal: $F_{2,8}$=0.64, *P*=0.53). Males treated with Aroclor 1242 had approximately 15-20% more spines on their dendrites in the HVC than males treated with Canola oil (Table 3.1). Males treated with PCB 52 had more spines on the proximal and distal portion of their dendrites, but approximately 20% fewer spines on the medial portion of their dendrites (Table 3.1).

In the RA, the number of spines in the proximal, medial, and distal portion of the dendrite did not differ significantly among treatments (Table 3.1; ANOVA: Proximal: $F_{2,11}$=0.08, *P*=0.93; Medial: $F_{2,9}$=0.36, *P*=0.70; Distal: $F_{2,6}$=1.26, *P*=0.30).

Discussion

A select number of studies have shown the consequences of environmental chemical pollution on the song nuclei and birdsong, yet with divergent results. DDT and its metabolites decrease HVC and RA volume in American robins (*Turdus migratorius*), presumably due to stress, neurotoxicity, and androgen receptor antagonism (Iwaniuk et al. 2006). Heavy metal pollution (including mercury pollution) has numerous affects on song, such as lower note diversity, shorter songs, and lower total amount of songs during the dawn chorus in Carolina wrens (*Thryothorus ludovicianus*), house wrens (*Troglodytes aedon*), song sparrows, and great tits (Gorissen et al. 2005; Hallinger et al. 2010). In contrast, Markman et al. (2008) reported that environmental estrogen exposure increased the HVC, song complexity, and female preference for song in European starlings (*Sturnus vulgaris*), although exposed males had reduced immune function. The results from this experiment coincide with the results from Markman et al. (2008) and contrasts previous findings that PCB-exposure results in decreased song nuclei volume (Hoogesteijn et al. 2008), contrary to our predictions.

Males treated with Aroclor 1242 sang less diverse songs and had more dendritic spines on HVC neurons than the males treated with the Canola oil control (Table 3.1). Aroclor 1242 is composed primarily of lower chlorinated congeners (Frame 1997), much like the profile of PCBs found in song sparrows with increased trill performance in the field (citation of Thesis: Chapter 1). Interestingly, Aroclor 1242 exposure on male zebra finch nestling seems to affect adult males similarly to environmental estrogens (Markman et al. 2008). Lower chlorinated PCBs, such as those in comprising Aroclor 1242, are known to have estrogenic actions (Plíšková et al. 2005), and at this level of exposure Aroclor 1242 seems to add to the masculinization of the HVC in the male brain (Arnold & Schlinger 1993). Because the HVC is the only song nucleus measured with

estrogen receptors (Fusani & Gahr 2006), it follows that Aroclor 1242 affects the neuronal morphology in this region the most.

Males treated with PCB 52 had more stereotyped songs as well, and more dendritic spines on the HVC neurons in the proximal and distal regions (Table 3.1). One interpretation of this decreased response to the pure estrogenic PCB-treatment in comparison to the males treated with Aroclor 1242 is that the combined exposure to the PCB congeners present in the Aroclor 1242 mixture, or a subset of those congeners, is what affected the HVC neuronal morphology and songs produced.

Unlike the song centers, PCB-treated males had fewer dendritic spines in the hippocampus than Canola-treated males (Table 3.1). Although the hippocampus contains estrogen receptors (Gahr et al. 1993), similar to the HVC, results indicate that developmental PCB-exposure significantly decreased the number of spines on all regions of the dendrites, with the exception of the medial portion in the PCB 52-treatment (Table 3.1). Because the hippocampus does have substantial numbers of corticosteroid receptors in mammals (Pryce 2008) and PCB-exposure does affect ligand binding in the hippocampus in rats (e.g. (Schantz et al. 1997)), it is not surprising that the zebra finch hippocampus was affected by the treatments. Yet, at this point it is not clear why the developmental PCB-exposure had an opposite effect on the hippocampal region than its effect on the HVC.

Although the PCB-treated males seem to have a positive song and cerebral song system response, the males were not as reproductively successful as the Canola treated males (citation of Thesis: Chapter 4). Therefore, it may be that an increased number of dendritic spines in the HVC and the decreased variability within a male's own song is not an indication of a higher quality song. Indeed, the language-related areas of the brain in humans contain more synapses at eighteen

months than when older (Huttenlocher 1990). Therefore, it may be that circuit differentiation through the attrition of spines is what is critical to the developing song system, and exposure to toxins during development is interfering with that process. In addition, the decreased motif diversity within PCB-treated males may not be an indication of higher quality song. It may be that decreased diversity constrains information that is communicated only through song variation (Sakata et al. 2008). Alternatively, it may be that PCB-treated males do actually produce higher quality songs (Sakata & Vehrencamp 2012) resulting from more dendritic spines in the HVC, but in a reproductive setting, another attribute of the individual besides song, such as the hippocampal size (Sherry et al. 1992) or immune function (Markman et al. 2008), is affecting their success.

This experiment adds new insights to the impact of sublethal levels of PCBs on developing birds, and clearly shows that PCB mixtures and pure PCB congeners do not result in equivalent changes in song characteristics or in neuron morphology in brain song nuclei. Further experimental investigation is needed into the effects of PCB-exposure on the developing hippocampus and song system to understand the mechanisms involved. In addition, the consequences of other congener mixtures should be explored to better comprehend the pressures on wild birds living in contaminated environments.

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Chapter 4:

THE EFFECT OF POLYCHLORINATED BIPHENYLS ON MALE ZEBRA FINCH REPRODUCTIVE SUCCESS AND BEHAVIOR

Abstract

Animals in the wild face many different forms of environmental stress, including exposure to chemical pollution. Sustained sublethal exposure to chemical pollutants during periods of development can have lasting consequences. Polychlorinated biphenyls (PCBs) are worldwide chemical pollutants that are known to cause many toxic effects at high exposure levels. At sublethal levels PCBs have been linked to various harmful effects, including disrupted reproduction and altered sexual behavior in female passerines. To date, tests for similar effects in male passerines have not been conducted. This study focuses on the effects of two PCBs, Aroclor 1242 (a commercial mixture) and PCB 52 (an estrogenic congener) on male zebra finch (*Taeniopygia guttata*) reproduction. Results indicate that although traditional measures of reproductive success are not altered by sublethal PCB exposure, both PCB forms do alter sexual behaviors and other reproductive parameters, such as increasing the number of nesting attempts before a clutch successfully produces at least one hatchling, increasing the numbers of eggs laid before the first successful clutch, and increasing the number of eggs buried. Instead of these effects being a consequence of compromised fertility in male zebra finches, behavioral differences between males seem to best explain these reproductive effects. Not only do the results highlight the difference between the effects of PCB mixtures and PCB congeners, these results confirm that sublethal PCB-exposure during development significantly alters important reproductive behaviors of adult male passerines.

Introduction

Wildlife populations face many environmental stressors. These stressors may be naturally occurring abiotic (e.g. food limitation (Hiom et al. 1991)) or biotic (e.g. competition (Dhondt 2012)) stress, or they may be anthropogenic (Acevedo-Whitehouse & Duffus 2009). A growing number of studies have examined anthropogenic disturbances as sources of stress to wildlife populations (Adams 2003; Munns 2006; Tarlow & Blumstein 2007), examining factors as varied as urban noise (Slabbekoorn & Peet 2003), habitat fragmentation (Kruess & Tscharntke 1994), and chemical pollution (Zala & Penn 2004). Like naturally occurring stressors, anthropogenic disturbances may be periodic, fluctuating in intensity seasonally (Eastwood & Couture 2002), or can be restricted to a certain time of the animals life, such as development (Dodson & Hanazato 1995).

Because organisms may be especially sensitive during development, anthropogenic stress experienced during the period of development may have especially harmful effects on the individual, with lasting consequences into adulthood. Indeed, the nutritional stress hypothesis postulates that adult morphology and behavior is of evolutionary significance because it indicates whether an individual received adequate nutrition during development (Nowicki et al. 1998; Trites & Donnelly 2003). Wildlife populations today not only face nutritional challenges during development, but also the challenge of exposure to chemically contaminated food during development (Holladay & Smialowicz 2000).

One problematic group of ubiquitous chemical pollutants, polychlorinated biphenyls (PCBs), were widely used last century in industry (Safe 1994). Due to the unsound disposal practices used, and their chemical stability, PCBs currently have been found in organisms at virtually every trophic level and in the most seemingly remote parts of the Earth (Brinkman & de

Kok 1980; Clarkson 1995; Safe & Hutzinger 1987). PCBs are a complex class of over 200 congeners with a di-benzene backbone and varying degrees of chlorination. Because industrial PCBs were produced as commercial mixtures (Frame 1997), animals in the wild are usually exposed to multiple congeners that exhibit diverse effects on biological systems (Fonnum et al. 2006). At high concentrations, PCBs can cause cancer and be fatal (Bertazzi et al. 1987). At sublethal concentrations, the position and number of the chlorines on the di-benzene backbone dictates their biological pathway (Van den Berg et al. 2006). PCBs with lower levels of chlorination have been linked to estrogenic actions, while higher chlorinated PCBs are linked to anti-estrogenic actions (Plíšková et al. 2005). Therefore, wildlife populations in PCB contaminated environments are likely encountering variable mixtures of PCBs, and each congener profile could be having distinct and diverse effects on various biological systems.

As environmental endocrine disruptors (Crews & Willingham 2000), PCBs have been shown to effect reproductive parameters in many organisms (e.g. minks (*Neovison vison*) (Aulerich & Ringer 1977), great black-backed gulls (*Larus marinus*) (Helberg et al. 2005), humans (*Homo sapiens*) (Lundqvist et al. 2006)). At the sublethal level, research on the reproductive effects of PCBs has focused on the female sexual partner. For example, PCB exposure has been correlated with female tree swallow (*Tachycineta bicolor*) plumage and clutch size in the field (McCarty & Secord 2000). Experimentally, PCB exposure to female zebra finches has affected the number of nests built, the number of clutches laid, and incubation time (Hoogesteijn et al. 2005). Additionally, although field studies indicate that sublethal PCB-exposure correlates with behavioral changes in birds (citation of Thesis: Chapter 2, (McCarty & Secord 1999a)), it is challenging to pinpoint the timing of exposure.

While studies to date show effects on reproductive behaviors from adult PCB-exposure,

we would also expect PCB-exposure on developing birds to have subsequent effects on reproductive behaviors. Exposure in contaminated areas is possible because adult passerines living in PCB-contaminated environments are likely to feed their offspring contaminated insects during the breeding season (Dauwe et al. 2003; Maul et al. 2006; Neigh et al. 2006; Park et al. 2009). Subsequent effects are likely to affect populations well beyond locations of contamination because natal dispersal in males is likely to move them from their nestling location (Clarke et al. 1997). While subsequent effects of adult exposure on female birds has been examined (Hoogesteijn et al. 2005), the existence of effects of natal PCB-exposure on male birds has not been the subject of experimental study.

This research provides the first test of the effects of natal exposure of PCBs on the reproductive behavior of an adult male bird. Ecologically relevant exposure to PCBs is replicated by dosing captive male zebra finch nestlings with environmentally relevant PCB congeners and mixtures, and observing their adult reproduction and behavior. Experimentally treated birds were exposed to one of two different PCB mixtures: an industrial PCB mixture, or a single PCB congener with known estrogen-like properties. The PCB mixture, Aroclor 1242, has approximately 42% chlorine by mass composition and is the primary industrial mixture that was released into the Hudson River in New York State last century (EPA 1997). The pure PCB treatment was PCB 52, a tetra-chlorinated PCB congener with known estrogenic properties (Jansen et al. 1993). We specifically test for the effects of this natal exposure on the reproductive ability and behaviors of these birds once they reach sexual maturity. This experiment attempts to add to our knowledge about the effect of PCBs on passerines, and to specifically examine whether developmental PCB-exposure affects male reproductive parameters.

Methods

Animals

All zebra finches involved in this study were from the colony of T. DeVoogd and housed in Uris Hall, Cornell University (Ithaca, New York, USA). Zebra finches were provided with Kaytee™ forti-finch diet, oyster shell (a grit and calcium source), cuttlebone (for calcium and beak maintenance), and water *ad libitum*. All individuals were banded as nestlings with a unique color band combination for identification (Avinet, Inc. Dryden, New York 13053-1103 USA). Day 1 for each chick was defined as the day it hatched. Subjects were housed in rooms on a 14L: 10D photoperiod, with the humidity maintained between 40-50%. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee (IACUC Protocol 1988-0135). All genetic procedures were performed in the Evolutionary Genetics Core Facility at Cornell University (Ithaca, New York 14853 USA).

Thirteen zebra finch pairs with prior breeding experience were housed in cages (60.96cm x 35.56cm x 40.64cm) equipped with one plastic nest cavity and coconut fiber nesting material. Cages were checked daily to monitor for nest building, incubation, and laying. Hatching order was recorded by marking each nestling daily with colored Crayola™ non-toxic permanent markers until day 14 when they were uniquely color banded for identification. These nestlings are defined as the F1 generation.

Genetic sexing

The sex of each F1 nestling was determined genetically following Qiagen DNeasy™ Blood & Tissue extraction protocol (QIAGEN, Catalogue number 69504, Valencia, California, USA) with sex subsequently confirmed by day 60 with examination of sex-specific adult plumage. One pinfeather, or 3-5 down feathers was taken to provide the needed DNA, and the

feather quill was submerged in buffer solution and lysed for 1-3 hours at 56ºC prior to extraction. The final elution volume was between 50-75µL. *Taq* PCR Kit (New England BioLabs® Inc., Catalogue number E5000S) was used for PCR amplification. Each 9.6µL PCR contained 2µL of template DNA, 5.9µL ddH_2O, 1µL 10x buffer, 0.4µL 50mM $MgCl_2$, 0.2µL 10mM dNTPs, 0.1µL Platinum *Taq*, and 0.2µL of each of the two 10µM W-allele primers (Kahn & Quinn 1998). The following PCR program was used for DNA amplification: an initial denaturation step of 90ºC for 2 minutes; followed by a 7-cycle touchdown: 94ºC for 50 seconds, 57-51ºC for 45 seconds (dropping 1ºC per cycle), 72ºC for 1 minute; another 30 cycles of 94ºC for 50 seconds, 50ºC for 45 seconds, then 72ºC for 1 minute; and a final hold temperature of 4ºC.

Experimental treatment

F1 nestlings were randomly assigned to one of three groups for experimental exposure to PCBs. The three groups were a control group, and two experimental groups each given a different PCB type. The dosage for each group was based on the only other known study of PCB dosage to zebra finches nestlings (Hoogesteijn 2003), although modified to more closely match what nestling may encounter in the environment in areas of PCB contamination (Echols et al. 2004; Maul et al. 2006; McCarty 2002; Secord et al. 1999). PCB solutions were administered using a 25 µL blunt-tipped Hamilton™ syringe by stimulating the gape reflex of the nestling with a slight touch, and drop feeding the solution into the gape. To prevent any cross-contamination, a separate syringe was dedicated to one of the three groups, and after every feeding session the syringe was flushed with hexane and wiped clean. The age at fledging was recorded, and the mass and left tarsus length was measured on day 20. F1 fledglings were placed in single-sex aviaries (91.44cm x 60.96cm x 121.92cm) in a separate room from their parents on day 51. On day 120 the mass and left tarsus length was measured again.

Canola oil (control) treatment

Individual F1 male nestlings assigned to the control group (N=13) were administered a total of 165µL of Canola oil across day 2 to day 8 of their lives (Table 4.1).

Aroclor 1242 treatment

Individual F1 male nestlings assigned to the Aroclor 1242 group (N=7) were administered a total of 165µL of Aroclor 1242 (AccuStandard.com) dissolved at 1-mg/mL in Canola oil over a 7-day period from day 2 to day 8 of the birds' lives (Table 4.1). Aroclor 1242 is a commercial PCB mixture that was the predominant mixture released on the Hudson River by General Electric in northeastern New York State last century (EPA 1997).

PCB 52 treatment

Individual F1 male nestlings assigned to the PCB 52 group (N=6) were administered 165µL of PCB 52 (AccuStandard.com) dissolved at a 1-mg/mL concentration in Canola oil across the 7-day period from day 2 to day 8 of life (Table 4.1). PCB 52 is a tetra-chlorinated PCB congener with chlorines at the 2, 5, 2', 5' positions around the di-benzene backbone. PCB 52 is known to have estrogenic properties (Jansen et al. 1993), and the chemical structure has estrogen receptor affinity (deCastro et al. 2006).

Table 4.1. Seven-day dosing regime from day 2 until day 8. Group 1 individuals received Canola oil. Group 2 and Group 3 individuals received Aroclor 1242 and PCB 52 respectively at a 1-mg/mL concentration dissolved in Canola oil.

	Canola oil group	**Aroclor 1242 group**		**PCB 52 group**	
Day Post-hatch	**Volume Canola oil (µL)**	**Solution volume (µL)**	**Aroclor 1242 (µg)**	**Solution volume (µL)**	**PCB 52 (µg)**
2	10	10	10	10	10
3	15	15	15	15	15
4	20	20	20	20	20
5	30	30	30	30	30
6	30	30	30	30	30
7	30	30	30	30	30
8	30	30	30	30	30

Experimental set-up

Thirteen identical breeding cages (60.96cm x 35.56cm x 40.64cm; Figure 4.1) were each equipped with one central perch spanning the length of the cage and two symmetrically placed shorter perches in the corners. Two identical nest boxes were placed at the same height and distance to cage corners and supplied with coconut fibre nesting material. The cages were visually, but not acoustically, separated from each other. The experiment began when all F1 males were a minimum of 100 days post-hatch. Groups of three adult zebra finches were placed in the experimental cages: one F1 male from the Canola oil group, either one F1 male from Aroclor 1242 group or one F1 male from the PCB 52 group, and one inexperienced and unrelated female with no PCB-exposure. Due to lack of space, there was no treatment with two F1 males from the Canola oil group and one inexperienced, unrelated, and unexposed female. For brevity, 'cage treatment' will refer to the treatment of the experimental male in the cage. When possible, the Aroclor 1242 (N=7) and the PCB 52 (N=6) experimental cages contained male pairs that were matched by family (i.e. male siblings were preferentially paired), size (weight and tarsus length), and age. Additionally, the color bands placed on the males as nestlings were removed, and the males were given a single black leg band uniquely placed to identified each male in the

experimental cages from their paired male and to prevent any preference for band color (Burley et al. 1982).

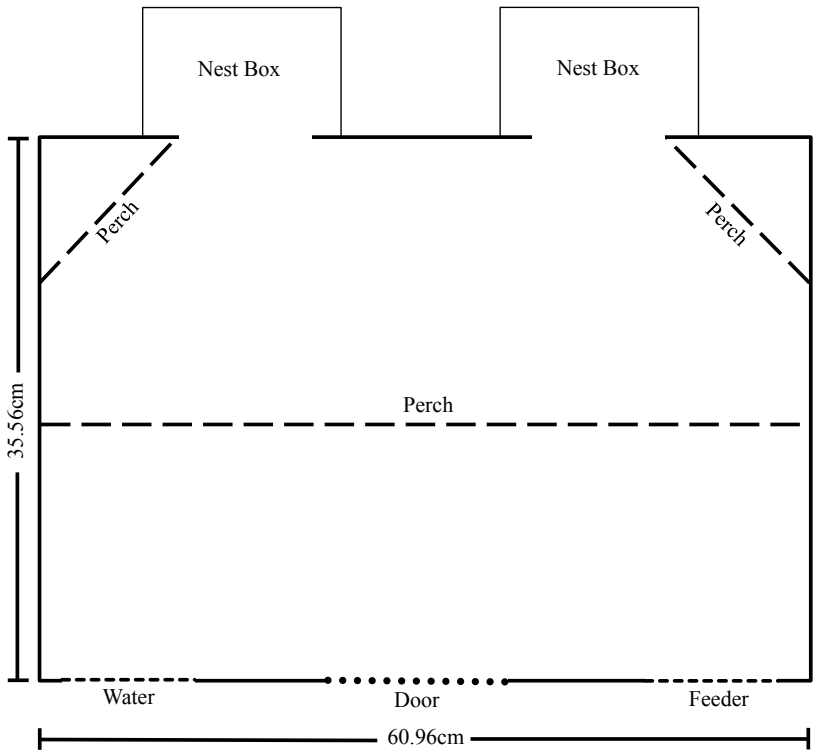

Figure 4.1. Top view of experimental breeding cages.

Behavioral observations

Ten-minute observation periods began on the second day of the experiment, and continued daily for either 90 days, or until 14 days after the last fledging event of the first successful clutch, whichever first occurred. The first successful clutch was defined as the first clutch within the 90-day period which fledged. Each cage was observed on every second day in a randomized order in the mornings within the first three hours of lights on, when the birds were most active.

Behavioral observations were grouped into three periods: pre-laying (before the first egg was laid), laying (after the first egg was laid, but before the first egg hatched, which includes the incubation period), and post-hatch (after the first egg hatched). During the pre-laying and laying

period the number of one-minute intervals where courtship behaviors (singing by the males, allopreening, and time in the nest box with the female) were observed at least once were recorded. During the pre-laying and laying period the number of ten-minute observations periods in which aggressive behavior (chasing another individual) were observed at least once were recorded. During the post-hatch period the number of one-minute intervals where courtship and parenting behaviors (singing by the males, allopreening, time in the nest box with the female, food provision to hatchlings) were observed at least once were recorded. During the post-hatch period the number of ten-minute observation periods in which aggressive behavior (chasing another individual) were observed at least once were recorded. In addition, the nest boxes in each cage were monitored daily and latency to nest building, the number of nests built, latency to laying, the numbers of eggs laid before a successful clutch, the number of clutches, clutch size, mortality, latency to fledging, fledging weight, and fledging tarsus length were recorded. Offspring from the F1 fathers are defined as F2 offspring.

Genetic parentage

The parentage of each F2 hatchling was genetically determined with microsatellites. Tissue samples were taken from F2 nestlings that died in the nest. Tissue samples were frozen at -20ºC immediately after collection. Between two and five pinfeathers were collected from living F2 fledglings for parentage analysis. The quills of the feather samples were stored submerged in buffer solution. Both tissue and feather samples were submerged in buffer solution and lysed for 1-3 hours at 56ºC prior to extraction and processed using Qiagen DNeasy™ Blood & Tissue extraction protocol (QIAGEN, Catalogue number 69504, Valencia, California, USA). The final elution volume was 75µL.

Six highly polymorphic microsatellites were selected based on non-overlapping size ranges, the absence of null alleles, and similar PCR programs from the set described in Forstmeier et al. (2007). Three primers were labeled using 6FAM fluorescent tags (Tgu12, Tgu9, and Tgu1), and three were labeled using NED fluorescent tags (Tgu4, Tgu3, and Tgu8). The QIAGEN Type-It Microsatellite Kit (QIAGEN, Catalogue number 206243) was used to perform a multiplex PCR amplification containing the six primer pairs. Each 25-µL PCR contained 12.5-µL of the 2x Type-it Multiplex PCR Master Mix, 2.5-µL 10x primer mix (containing 2µM each primer), 2-µL template DNA, and 8-µL ddH_2O. The PCR program had an initial denaturing step at 95ºC for 5 minutes, followed by a 5-cycle touchdown: 94ºC for 30 seconds, 60-56ºC for 90 seconds (dropping 1ºC per cycle), 72ºC for 30 seconds; another 23 cycles of 94ºC for 30 seconds, 56ºC for 90 seconds, 72ºC for 30 seconds; and a final extension at 60ºC for 30 minutes.

The PCR products were diluted 1:10 with ddH_2O (2-µL PCR product and 18-µL ddH_2O). Then 2µL of the 1:10 PCR-product dilution was combined with 18-µL of HI-DI formamide and 0.3-µL GS500 LIZ 250 base pair size standard and sequenced on an Applied BioSystems 3730xl Genetic Analyzer. The raw data was analyzed using Genemapper 4.0 software.

Statistical analysis

Excluded from the analysis are the cages in which an adult male died. For the courtship behaviors, each ten-minute observation period was divided into ten one-minute intervals. If a courtship behavior occurred at any time during the one-minute interval, the bird was scored as performing that behavior during that minute, for a score of one. For the aggressive behaviors, each ten-minute observation period was considered the unit of measurement. If an aggressive behavior occurred at any time during the ten-minute observation period, the bird was scored as performing that behavior during that observation period, for a score of one. The daily scores for

courtship and aggressive behavior were summed for each F1 male during each behavioral period (pre-laying, laying, post-hatch). The behavioral data were analyzed as count data, proportional data (the time an individual performed a behavior/total time that behavior was performed in the cage), or when there was an excess of zeroes, binary data. Count and proportion data were analyzed using an ANOVA or t-test for normally distributed data, and the non-parametric Mann-Whitney test for data with non-normally distributed residuals. Binary data were analyzed using a Chi-squared test, or a Pearson's Chi-squared test. When the sample size was small, a Fisher's exact test for was used. Data are reported as mean±SE, and all tests are considered significant when $P<0.05$. Statistical analysis were performed with JMP® 9.0.2.

Results

There was no difference between the F1 male treatment groups in fledging age, body mass, or tarsus length in measurements taken at day 20, day 120, and at the start of the experiment (Table 4.2). There was also no difference in body mass or tarsus length between the adult females in the two types of treatment cages (Table 4.2).

Table 4.2. Size and fledging age of the F1 generation males and females. An ANOVA tested the difference between F1 male treatment groups for average fledging age, body mass, and tarsus length at day 20, day 120, and on the first day of the experiment (±SE). A Mann-Whitney test was used to determine differences in F1 female size between the two treatment cages.

	Treatment	N	Mean Fledgling Age	Mean Day 20 Mass (g)	Mean Day 20 Tarsus Length (mm)	Mean Day 120 Mass (g)	Mean Day 120 Tarsus Length (mm)	Mean Exp. Mass (g)	Mean Exp. Tarsus Length (mm)
F1 Males	Canola	13	19.71 (±0.36)	11.64 (±0.34)	14.43 (±0.19)	13.36 (±0.64)	15.07 (±0.25)	13.93 (±0.38)	14.53 (±0.19)
	Aroclor 1242	7	19.77 (±0.32)	12.12 (±0.19)	14.26 (±0.22)	13.46 (±0.29)	14.92 (±0.19)	14.04 (±0.31)	14.31 (±0.21)
	PCB 52	6	19.5 (±0.34)	11.58 (±0.30)	13.75 (±0.13)	13.17 (±0.44)	14.51 (±0.20)	13.33 (±0.36)	14.12 (±0.14)
ANOVA	F		0.1377	1.3505	1.4035	0.1129	1.3581	0.6840	0.7308
	P		0.8721	0.2805	0.2687	0.8937	0.2770	0.5187	0.4924
F1 Females	Canola-Aroclor 1242 cages	7						15.00 (±0.53)	14.29 (±0.25)
	Canola-PCB 52 cages	6						14.17 (±0.49)	13.87 (±0.23)
Mann-Whitney	*P*							0.3119	0.1979

Behavioral characteristics

Pre-laying period

During the pre-laying period, the three pairwise adult aggressive interactions were not different between the two types of cage treatments (all Pearson's Chi-square tests: $P>0.05$). Within the two PCB treatment groups, there was no difference between the amounts of aggression observed between the three adults (all t-tests: $P>0.05$). There was also no difference in the proportion of time either male in either cage treatment spent allopreening with the female (Mann-Whitney U test: Aroclor 1242 cages: U=-0.60, $P=0.55$; PCB 52 cages: U=-0.74, $P=0.46$) or was in the nest box with the female (Mann-Whitney U test: Aroclor 1242 cages: U=0.54, $P=0.59$; PCB 52 cages: U=-1.07, $P=0.28$).

Laying period

During the laying period, there was no difference between the two types of treatment cages in the probability of presence or absence of aggression between each of the three adults (all Pearson's Chi-square tests: $P>0.05$), except for female aggression toward the Canola male in the Aroclor 1242 cages (Figure 4.2a). Between the two PCB treatments, a larger proportion of females in Aroclor 1242 cages exhibited aggression towards the Canola males than the proportion of females in the PCB 52 cages that showed aggression towards the Canola males (Figure 4.2a, Pearson's Chi-square test: $X^2_1=6.96$; P=0.008). In the Aroclor 1242 cages, the females displayed more aggression towards the Canola males than did females towards Canola males in the PCB 52 cages (Figure 4.2b, Mann-Whitney U test: U=-2.37, P=0.018). Overall, the females were more aggressive in the Aroclor 1242 cages than the PCB 52 cages (Figure 4.2c, ANOVA: $F_{2,9}=9.12$, P=0.012). Within the PCB 52 cages, the proportion of Canola males that showed aggression was greater than the proportion of PCB 52 males that showed aggression (Pearson's Chi-square test: $X^2_1=4.00$; P=0.046).

In the PCB 52 cages the Canola males spent a significantly larger proportion of time with the females in the nest box during the laying period than the PCB 52 males (Mann-Whitney U test: U=-2.19, P=0.028). Furthermore, building nests over eggs was observed more frequently in the Aroclor 1242 cages (7/7) than in the PCB 52 cages (2/6) (Fisher's exact test: P=0.02).

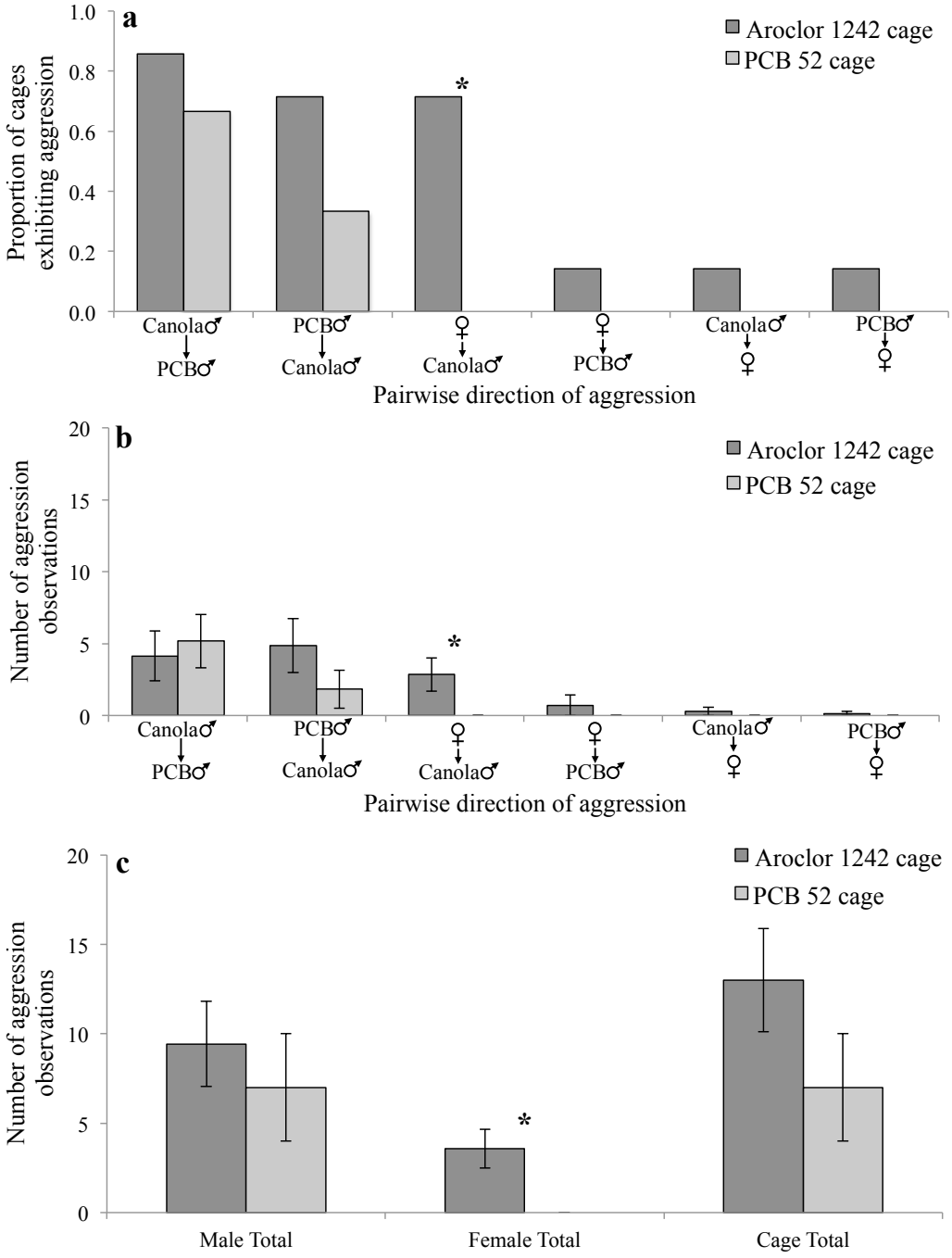

Figure 4.2. Aggressive behavior during the laying period. **a)** The proportion of cages exhibiting pairwise aggression. Females in the Aroclor 1242 cages exhibited more aggression towards the Canola males than the aggression of the females in the PCB 52 towards the Canola males (Pearson's Chi-square test: X^2_1 =6.96; P=0.008). **b)** The average number of pairwise aggression observations (±SE) exhibited by the two types of treatment cages. Female aggression towards the Canola male was greater in the Aroclor 1242 cage than in the PCB 52 cage (Mann-Whitney U test: U=-2.37, *P*=0.018). **c)** The average total of aggression observations (±SE) observed in the two cage treatments. Females were, overall, more aggressive in the Aroclor 1242 cages than the PCB 52 cages (ANOVA: $F_{2,9}$=9.12, *P*=0.012).

Post-hatch period

During the post-hatch period, there was no difference between the two PCB treatments in who was in the nest box with the female (Fisher's exact test: P>0.05). In comparisons of experimental males exposed to the two PCB treatments, the Aroclor 1242 males were in the nest box with the female significantly more frequently than the PCB 52 males (Figure 4.3, Mann Whitney U test: U=-2.01, *P*=0.04). Within the cages, the Canola males were in the nest box with the female significantly more in the PCB 52 cages than the Canola males in the Aroclor 1242 cages (Figure 4.3, Mann Whitney U test: U=-2.01, *P*=0.04). There was no difference in the occurrence of feeding offspring (Fisher's exact test: P>0.05) or the proportion of offspring feeding (Mann-Whitney U test: *P*>0.05) during the post-hatch period either between PCB treatments or between Canola and PCB males.

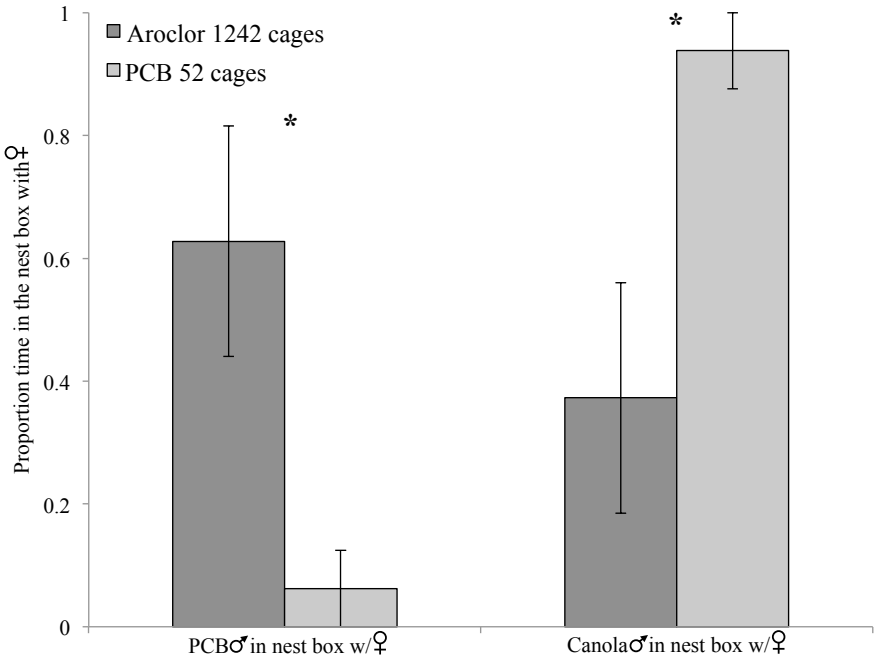

Figure 4.3. The average proportion of time (±SE) that each male spent in the nest box with the female during the post-hatch period. The Aroclor 1242 males were in the nest box with the female significantly more than the PCB 52 males (Mann Whitney U test: U=-2.01, *P*=0.04). The Canola males were in the nest box with the female significantly more in the PCB 52 cages than in the Aroclor 1242 cages (Mann Whitney U test: U=-2.01, *P*=0.04).

Reproductive characteristics

There were no differences in any traditional measures of reproductive success between the F1 PCB-treated and Canola-treated males, either with both PCB treatments' data combined or the two PCB-treatments (Aroclor 1242 and PCB 52) used as separate groups, in comparisons between males and their Canola-treated paired control males (Table 4.3). The F1 genetic parentage had no significant effect on the age at fledging, mass at fledging, or tarsus length at fledging of the F2 offspring (Table 4.4). The Aroclor 1242 cages (average 2.2) differed significantly from the PCB 52 cages (average 1.2) in the number of clutches laid before the first successful clutch (Figure 4.4a, t-test: t_{12}=2.36, *P*=0.046). In addition, females in the Aroclor 1242 cages laid a significantly greater number of eggs (average 7.2) before their first successful clutch than the females in the PCB 52 cages (average 1.6) (Figure 4.4b, t-test: t_{10}=3.08, *P*=0.01). The two cage treatments did not differ significantly in their hatching success (Figure 4.4c; number of eggs hatched/ successful clutch size; Aroclor 1242 cages=0.57; PCB 52 cages=0.56; t-test: t_{12}=0.04, *P*=0.97) or in their fledging success (Figure 4.4d; number of eggs fledged/ successful clutch size; Aroclor 1242 cages=0.40; PCB 52 cages=0.56; t-test: t_{12}=-0.75, *P*=0.48).

Table 4.3. Reproductive success. Results of differences between PCB-treated and Canola-treated males, or between the individual PCB-treated (Aroclor 1242 and PCB 52) F1 males and their Canola male pairs.

Clutch	Grouping	Response	Predictor	Statistics	P-value
1st clutch	F1 PCB-treated males vs. F1 Canola males	Presence/ absence F2 fledglings	Genetic parentage	Pearson's Chi-squared: X^2_1 =0.867	0.3519
	F1 PCB-treated males vs. F1 Canola males	Clutch size	Published values (Frith & Tilt 1959; Zann 1994)	t-test: t_1=1.79	0.1114
	F1 PCB-treated males vs. F1 Canola males	% fledglings/ clutch	Published values (Frith & Tilt 1959; Zann 1994)	t-test: t_1=0.75	0.4759
	F1 Aroclor 1242 males vs. F1 Canola males	Number of fledglings	Genetic parentage	Mann-Whitney	0.3914
	F1 Aroclor 1242 males vs. F1 Canola males	Presence/ absence F2 fledglings	Genetic parentage	Fisher's exact	1.000
	F1 PCB 52 males vs. F1 Canola males	Number of fledglings	Genetic parentage	Mann-Whitney	0.3239
	F1 PCB 52 males vs. F1 Canola males	Presence/ absence F2 fledglings	Genetic parentage	Fisher's exact	1.000
1st successful clutch	F1 PCB-treated males vs. F1 Canola males	Presence/ absence F2 fledglings	Genetic parentage	Pearson's Chi-squared: X^2_1 =0.650	0.4201
	F1 PCB-treated males vs. F1 Canola males	Clutch size	Genetic parentage	Mann-Whitney	0.5187
	F1 Aroclor 1242 males vs. F1 Canola males	Number of fledglings	Genetic parentage	Mann-Whitney test	0.3336
	F1 Aroclor 1242 males vs. F1 Canola males	Presence/ absence F2 fledglings	Genetic parentage	Fisher's exact	0.5594
	F1 PCB 52 males vs. F1 Canola males	Number of fledglings	Genetic parentage	Mann-Whitney	0.7965
	F1 PCB 52 males vs. F1 Canola males	Presence/ absence F2 fledglings	Genetic parentage	Fisher's exact	0.5594

Table 4.4. Size and fledging age of the F2 generation males and females. An ANOVA tested the difference between F2 offspring for average fledging age, body mass and tarsus length at day of fledgling (±SE).

Treatment	F1 Genetic Father	N	F2 Fledgling Age (days)	F2 Mass at Fledging (g)	F2 Tarsus Length at Fledging (mm)	F2 Males	F2 Females	Dead in Nest
Canola-PCB 52	**Canola**	7	19.0 (±0.53)	10.71 (±0.29)	14.01 (±0.28)	3	4	0
	PCB 52	5	19.2 (±0.37)	10.90 (±0.24)	13.83 (±0.19)	1	4	0
Canola-Aroclor 1242	**Canola**	5	18.2 (±1.20)	11.40 (±0.19)	14.27 (±0.22)	3	2	2
	Aroclor 1242	11	20.9 (±0.56)	11.27 (±0.48)	13.93 (±0.16)	5	6	6
ANOVA	**F**		3.143	0.1353	0.53886			
	P		0.0986	0.875	0.6056			

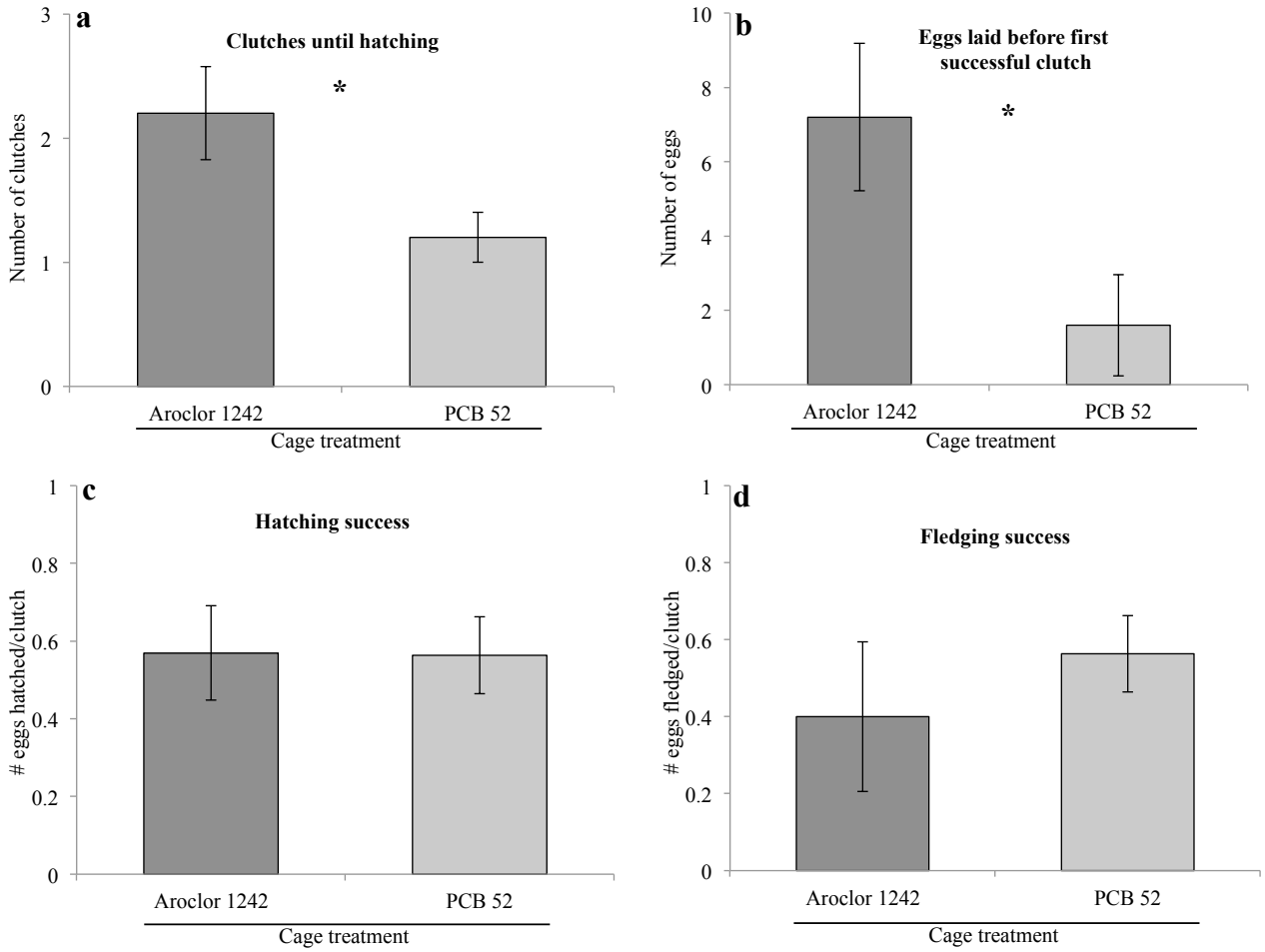

Figure 4.4. Reproductive characteristics of the two cage treatment types. **a)** The average number (±SE) of clutches laid in each treatment cage before the first successful fledging clutch (t-test: t_{12}=2.36, *P*=0.046). **b)** The average number of eggs laid (±SE) in each treatment cage before the first successful clutch was laid (t-test: t_{10}=3.08, *P*=0.01). **c)** The average hatching success (number of eggs hatched/ successful clutch size) (±SE) in each treatment cage (t-test: t_{12}=0.04, *P*=0.97). **d)** The average fledging success (number of eggs fledged/ successful clutch size) (±SE) in each treatment cage (t-test: t_{12}=-0.75, *P*=0.48).

Discussion

Developmental exposure to PCB 52 resulted in a shorter latency to successful clutches in comparison to the Aroclor 1242 treatment cages. Within the PCB 52 treatment cages, the behavioral data revealed that the females prefer the Canola males to the PCB 52 males, and there was no observed aggression between the males and female. This less aggressive atmosphere appeared to facilitate a shorter latency to successful clutches.

In contrast, developmental exposure to sublethal levels of Aroclor 1242 affected reproduction by delaying the latency to successful clutches in male zebra finches. Behavior may have mediated this result through adults in the Aroclor 1242 treatment cages showing more aggression, resulting in the female not clearly socially pairing with either the Aroclor 1242 or the Canola male in their cage. Instead, females in the Aroclor 1242 treatment cages were aggressive towards both males, and that both males were aggressive towards her as well (Figure 4.2). In addition, the females were observed spending similar amounts of time in the nest box with the Aroclor 1242 male and the Canola male within the Aroclor 1242 cage treatment (Figure 4.3). Therefore, the delay in the first successful clutch in the Aroclor 1242 cage treatment, an event important in reproductive success in a number of species (for examples see Ingold (1996) and Siikamäki (1998)), seems to be mainly a result of high levels of aggression during the laying period both between the males and between the female and the males.

The male developmental PCB dosage resulted in behavioral differences between the cage treatments indicating there were differences in social interactions that influenced the reproductive characteristics between the two PCB-treatment groups. Although the reproductive characteristics results initially appear to indicate the Aroclor 1242 cage treatment was, overall, more disruptive than the PCB 52 cage treatment, the behavioral results indicate a more complicated story. One explanation is that environmental PCB-exposure of estrogen mimicking PCBs during development, such as PCB 52, impacts adult male behavior, and results in males that are less aggressive and less able to secure female partners. In contrast, the developmental exposure to Aroclor 1242 increases aggression in a social context, resulting in more failed clutches. Therefore, environmental PCB-exposure to male passerines during development is likely to impact their adult behavior, with severe reproductive consequences.

This experiment found no evidence that the dosage regime administered to male zebra finches nestlings affected their morphological development (Table 4.2), or the hatching success of eggs in the nest of their partner (Table 4.3). The number of hatchlings in the first successful clutch, the number of fledglings in the first successful clutch, fledgling success, and hatching success all did not differ between the treatment groups (Table 4.3, Figure 4.4). Additionally, because the females were untreated, and the paternity data indicated that each male F1 treatment group sired offspring (Table 4.4), there is no indication that the PCB-treatments caused infertility. Because parentage of the unhatched eggs was unknown, there are no measures of the relative fertility of the males. However, the treatment groups did differ in other facets of reproduction. Females in cages with Aroclor 1242 treated males needed to lay more clutches and more eggs in order to raise any young, and hence would have a reduced fitness in the wild (Figure 4.4).

This behavioral breeding experiment in zebra finches adds to the body of literature (e.g. (Bonefeld-Jorgensen et al. 2001; Galassi et al. 2002), showing that pure PCB congeners and PCB mixtures can have contrasting effects on biological systems. Furthermore, this experiment clearly shows that exposure of males to PCBs during development influences their adult behavior in ways that can have drastic consequences on their reproduction, and these consequences are different based on the suite of PCB-congeners to which birds are exposed. However, more research is required to understand how the specific PCB congener(s) influenced development in such a way as to alter adult behavior. Therefore, it is imperative that toxicological studies consider the timing, duration, and amount of exposure, but also consider which specific congeners, or mixture of congeners, are involved in the birds' exposure. Furthermore, toxicological research is needed to consider a broad suite of effects of exposure to toxins. This study is novel in that is shows that sublethal natal exposure of male birds to PCBs affects

reproductive behaviors of these birds as adults or impacts the behaviors and/or breeding success of their reproductive partners. Yet, some measures of reproductive success were not affected by our experimental PCB treatments. Therefore, future research needs to examine an expanded suite of response variables in order to attain a more complete picture of the consequences of sublethal PCB-exposure during development on male zebra finches.

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Chapter 5:

EARLY EXPOSURE OF POLYCHLORINATED BIPHENYLS ON FEMALE ZEBRA FINCHES INFLUENCES SONG PREFERENCES

Abstract

Polychlorinated biphenyls (PCBs) are industrial environmental toxins and known endocrine disruptors. Exposure to sublethal levels of PCBs during development has been shown to have many influences on subsequent growth and adult behavior. This study investigates the consequences of PCB-exposure during development on female zebra finch (*Taeniopygia guttata*) size on adult female song preference. Although PCBs have been previously shown to decrease areas in the song system important in song learning and production in both male and female zebra finches (*Taeniopygia guttata*), female perception of PCB-exposed male song, and the effect of PCB-exposure on female song preferences has not yet been explored. Female zebra finch nestlings were exposed to 165µg total of Aroclor 1242 or PCB 52 from day 2 until day 8, measured at day 20 and day 120, and were given a song choice assay starting at approximately 365 days. Results show that PCBs do not appear to alter female size at day 20 or at day 120, or fledging age. The results of the song choice trials show that PCB-exposure during development does appear to effect female song preference, with females treated with Aroclor 1242 showing the strongest song preferences. These results highlight the difference between the effects of PCB mixtures and PCB congeners, and show that sublethal PCB-exposure during development can have important effects on adult female passerine behavior.

Introduction

Polychlorinated biphenyls (PCBs) are worldwide chemical pollutants that were first manufactured in the United States in 1929 (Hutzinger et al. 1979; Waid 1986). Although PCB manufacturing in the United States was banned fifty years later, they continue to be a serious environmental threat today (Blais et al. 2006; Henshel & Sparks 2006). Due to their widespread application and unsound disposal practices, it is estimated that one-third of the total PCBs manufactured in the United States have since entered the environment and have been found in organisms at virtually every trophic level (Brinkman & de Kok 1980; Clarkson 1995; Safe & Hutzinger 1987).

PCBs have between one and ten possible chlorination positions, allowing for over 200 PCB congeners, which display a wide range biological effects (Fonnum et al. 2006). Higher chlorinated PCBs are known to be more stable, less likely to be metabolized, and more toxic (Van den Berg et al. 2006), while coplanar PCBs (unchlorinated at the 2, 2', 6, 6'- *ortho* position) have a flatter configuration and toxicity similar to dioxin (Van den Berg et al. 2006). Additionally, PCBs influence estrogen and androgen receptor activity (Bonefeld-Jorgensen et al. 2001), and many endocrine and reproductive effects of PCB-exposure have been reported (Ax & Hansen 1975; Barron et al. 1995; Bonefeld-Jorgensen et al. 2001; Giesy et al. 2003). Specifically, lower chlorinated PCBs are associated with estrogenic actions, and higher chlorinated PCBs and PCB metabolites are associated with anti-estrogenic actions (Plíšková et al. 2005). Commercially in the United States PCBs were produced as mixtures, with the trade name Aroclor. So, for example, Aroclor 1256 is a mixture of PCBs with 56% chlorine by mass. Therefore, wildlife exposure to PCBs also is not to just one specific congener, but to a mixture of congeners.

Early nutrition has been shown to be an important factor in development (Grantham-McGregor 1995), with consequences lasting into adulthood and effecting survival and reproductive success (Metcalfe & Monaghan 2001; Searcy et al. 2004). Yet, in today's environment, many developing young do not only have early nutritional challenges to contend with, but are also exposed to environmental pollutants like PCBs. The complexity of PCBs as a class of chemicals signifies that exposure to specific congeners, as well as the timing and quantity of the exposure, are all necessary factors to consider when investigating the consequences of developmental PCB exposure. PCB exposure during development results in a range of responses spanning from morphological effects (Gutleb et al. 2000), to altered reproductive behavior effects in the adult (Hoogesteijn et al. 2005).

One vital aspect of reproductive success in adult female passerines is mate choice. Female mate choice is a strong selective force (Andersson 1994), and from the females' perspective, her mate choice decision is crucial to her reproductive success. Although studies investigating the factors contributing to mate choice indicate that a females' early social environment is crucial to her adult mate preferences (Lauay et al. 2004; Riebel et al. 2009), theory predicts that her physical environment should be equally important (Nowicki et al. 1998). Therefore, exposure to sublethal levels of endocrine disruptors, such as PCBs, during development may not only influence her physical development, but also is likely to influence her adult mate preferences. In addition, male bird song is a hormonally controlled behavior that is also likely to be influenced by sublethal levels of PCBs. Research has shown that the robust nucleus of the arcopallium (RA, involved in individual note production) is reduced in volume when both male and female captive zebra finches (*Taeniopygia guttata*) were exposed to Aroclor 1248, a highly chlorinated PCB mixture, during development (Hoogesteijn et al. 2008). Yet when exposed to Aroclor 1242, a lower

chlorinated PCB mixture, during development, male zebra finches reproduce their song more accurately, and have an increased number of HVC dendritic spines (citation of Thesis: Chapter 3). As a result, PCB-exposure during development may be affecting both male song characteristics that may alter female song preferences, as well as female song perception. For example, male European starlings (*Sturnus vulgaris*) that have been exposed to environmental estrogen mimics have more complex songs and are preferred by females (Markman et al. 2008). Therefore, it is likely that exogenous compounds that mimic the action of hormones, like PCBs, effect females response to song (LeBlanc et al. 2007).

This study aims to elucidate the effects of early sublethal PCB-exposure on physical development, as well as female song discrimination abilities and song preferences. The three main objectives of this experiment are: 1) to determine whether sublethal PCB-exposure during development effects female size and/or fledging age, 2) to determine whether female zebra finches have a preference for songs from PCB-treated or untreated males, and 3) to determine whether PCB-treated female zebra finches have different song preferences than untreated females.

METHODS

Animals

All zebra finches involved in this study fed with Kaytee™ forti-finch diet, and given oyster shell (a grit and calcium source), cuttlebone (for calcium and beak maintenance), and water *ad libitum*. Aviaries (91.44cm x 60.96cm x 121.92cm) and breeding cages (60.96cm x 35.56cm x 40.64cm) were equipped with multiple perch sizes, and individuals were banded with a unique color band combination (Avinet, Inc. Dryden, New York, 13053-1103, USA) for individual identification. The day each chick hatched was designated as its day 1. Bird rooms were on a 14L:

10D photoperiod and the humidity was maintained between 40-50%. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee (IACUC Protocol 1988-0135).

Experimental treatment groups

Thirteen experienced zebra finch pairs were caged in breeding cages equipped with one plastic nest cavity and coconut fiber nesting material. Cages were checked daily to monitor nest building, laying, incubation, and hatching order. Hatching order was recorded and each nestling was uniquely marked with Crayola™ non-toxic permanent markers until uniquely color banded at day 14. All nestlings were sexed genetically and confirmed with adult plumage. A solitary pinfeather, or 3-5 down feathers was used as genetic material. Qiagen DNeasy™ Blood & Tissue Kit (QIAGEN, Catalogue Number 69504) was used to extract the DNA and prepare the samples for the PCR process. Feathers were lysed for 1-3 hours at 56ºC prior to extraction and the final elution volume was 50-75µL. PCR amplification was performed using *Taq* PCR Kit (New England BioLabs® Inc., Catalogue number E5000S). Each 9.6µL PCR contained 2µL template DNA, 5.9µL ddH_2O, 1µL 10x buffer, 0.4µL 50mM $MgCl_2$, 0.2µL 10mM dNTPs, 0.1µL Platinum *Taq*, and 0.2µL of each of the two 10µM W-allele primers (Kahn & Quinn 1998). The following PCR program was used for DNA amplification: an initial denaturation step of 90ºC for 2 minutes; followed by a 7-cycle touchdown: 94ºC for 50 seconds, 57-51ºC for 45 seconds (dropping 1ºC per cycle), 72ºC for 1 minute; another 30 cycles of 94ºC for 50 seconds, 50ºC for 45 seconds, then 72ºC for 1 minute; and a final hold temperature of 4ºC. All genetic procedures were performed in the Evolutionary Genetics Core Facility at Cornell University (Ithaca, New York 14853 USA).

Both female and male nestlings were randomly assigned to an experimental treatment group. The dosage period was chosen to maximally overlap with the timing of HVC and RA cell

division (DeVoogd, personal communication). Dosage amount was based on the only other known study of PCB dosage to zebra finches nestlings (Hoogesteijn 2003) and modified to more closely match what nestling may encounter in areas of PCB contamination (Echols et al. 2004; Maul et al. 2006; McCarty 2002; Secord et al. 1999). The solutions were administered using a 25 µL blunt-tipped Hamilton™ syringe by stimulating the gape reflex of the nestling with a slight touch, and drop feeding the solution into the gape. To prevent any cross-contamination, a syringe was dedicated to one of the three groups (Canola oil, Aroclor 1242, or PCB 52), and after every feeding session, the syringe was flushed with hexane and wiped clean. The age at fledging was recorded, and the mass and left tarsus length was measured on day 20. Fledglings were placed in single-sex aviaries in a separate room from their parents on day 51. On day 120 the mass and left tarsus length was measured again.

Canola oil control group

Individual nestlings assigned to the control group (female N=7, male N=10) were administered a total of 165µg of Canola oil from day 2 to day 8 in a 7-day regiment (Table 5.1).

Table 5.1. Seven-day dosing regime of the Canola oil group, the Aroclor 1242 group, and the PCB 52 group. Canola oil individuals received only Canola oil. Individuals from the Aroclor 1242 group and the PCB 52 group received Aroclor 1242 and PCB 52 respectively at a 1-mg/mL concentration dissolved in Canola oil.

	Canola oil group	**Aroclor 1242 group**		**PCB 52 group**	
Day post-hatch	**Volume Canola oil (µL)**	**Solution volume (µL)**	**Aroclor 1242 (µg)**	**Solution volume (µL)**	**PCB 52 (µg)**
2	10	10	10	10	10
3	15	15	15	15	15
4	20	20	20	20	20
5	30	30	30	30	30
6	30	30	30	30	30
7	30	30	30	30	30
8	30	30	30	30	30

Aroclor 1242 group

Individuals assigned to the Aroclor 1242 experimental group (female N=6, male N=4) were administered a total of 165µg of Aroclor 1242 (AccuStandard.com) from day 2 to day 8 in a 7-day regiment (Table 5.1). The Aroclor 1242 was mixed with Canola oil to produce a 1-mg/mL solution. Aroclor 1242 is a PCB mixture containing approximately 42% chlorine by mass. This mixture was the predominant mixture released on the Hudson River in northeastern New York State by General Electric last century (Hudson River Natural Resource Trustees 2005).

PCB 52 group

Individuals assigned to the PCB 52 experimental group (female N=7, male N=6) were administered a total of 165µg of PCB 52 (AccuStandard.com) from day 2 to day 8 in a 7-day regiment (Table 5.1). The PCB 52 was mixed with Canola oil to produce a 1-mg/mL solution. PCB 52 is an isolated PCB congener, with four chlorines at the 2, 5, 2', 5' positions around the di-benzene backbone. PCB 52 has a chemical structure associated with affinity at the estrogen receptor (deCastro et al. 2006), and has estrogenic properties (Jansen et al. 1993).

Stimulus recordings

Females were presented with three sets of trials, where each trial was a choice between two recordings. The first trial type was a choice between a 'social' male zebra finch song (N=4) and an 'isolate' male zebra finch song (N=4). The 'social' male song was from zebra finches raised with a tutor, and the 'isolate' song was from zebra finches raised in isolation, without a tutor. The first trial type was from archived recordings of males previously housed in the DeVoogd laboratory (Uris Hall, Cornell University, Ithaca, New York 14853 USA), and untreated female zebra finches had previously shown to be able to distinguish between these two song treatments in this experimental set-up (Lauay et al. 2004). The second trial type was a choice

between the song from a male treated with Canola oil and a song from a male treated with Aroclor 1242. The third trial type was a choice between the song from a male treated with Canola oil and the song from a male treated with PCB 52. All paired choices were corrected for by family, so that no female was presented with the song of a family member. Furthermore, if there were male siblings from different treatment groups, their songs were paired in the trials. The males treated with Canola oil, Aroclor 1242, and PCB 52 were recorded on day 120 (± 1 day). Each recording session lasted for one hour. The same experienced, unrelated, adult female was used as a stimulus in all song recordings. Songs were recorded with a Tascam HD-P2 Recorder, a Universal Telinga Pro 24-inch Parabola, and a Sennheiser ME62 Omni Microphone. Recordings were made directly onto a 4GB flash card in a sound proof room. The recordings from all males were analyzed with Sound Analysis Pro (http://ofer.sci.ccny.cuny.edu/sound_analysis_pro) and the results of that analysis are reported elsewhere (citation of Thesis: Chapter 3).

Song clips were generated using RavenPro 1.4 ((Bioacoustics Research Program, Cornell University) and Audacity software (http://audacity.sourceforge.net/). The individual motif (song type) of each male was identified, and bouts (repetitions of the motif) were created in a standardized fashion by making four copies of the motif, then generating 1 second of silence. The standardized bouts of each male in the pair were alternated within a track, and repeated to make a 2-minute track. Each trial consisted of two tracks, with each individual male being presented first, and with the individuals coming from different speakers.

Experimental song trials

Adult female subjects (N=20) began trials when approximately 365 days old, and continued them for approximately 6 months. All song trials were performed in the mornings from 0700-1100, the observer was blind to the subjects group, and the trial protocol was performed

after Lauay et al. (2004). The playback volume was measured from the center of the experimental cage set-up (Figure 5.1) before each trial to ensure that the sound level was 60-65 dB (the volume level in the housing rooms). The female subject placed in the center cage of the experimental apparatus and given 10 minutes to acclimate to the cage before each trial. After the acclimation period, Track 1 was played (2 minutes) while the subject remained in the center cage (cage 3). Then sliding trapdoors opened (Figure 5.1) and the females' location was observed and recorded for 8 minutes. Since the subjects often perched on the threshold of the cages, the females were giving a score of 1 for a time interval if her whole body, or if her head was in the cage. After the trial period the subject was returned to the center cage, the trapdoors were shut, and the procedure repeated for with the songs from the two males originating from the opposite speaker.

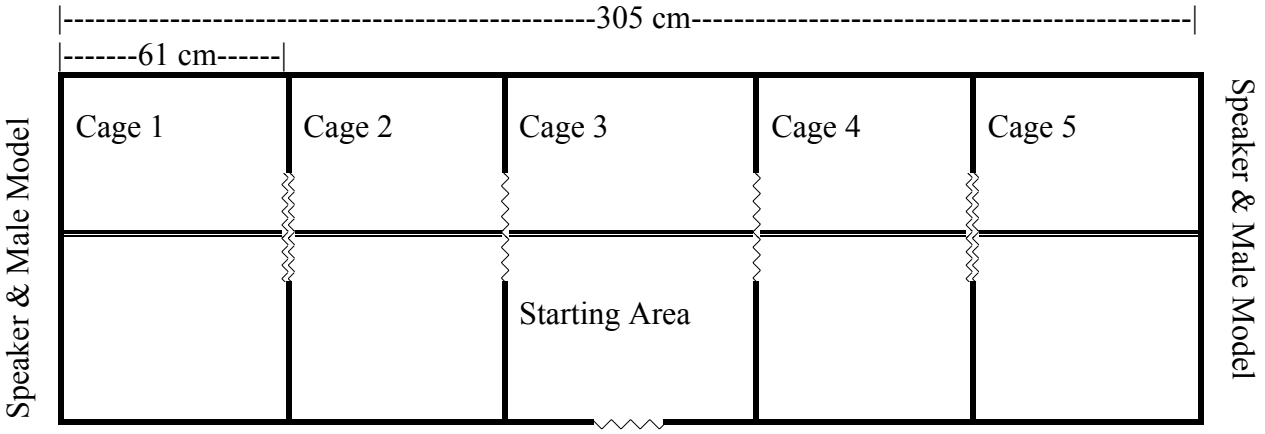

Figure 5.1. Top view of the experimental cage. Dark lines indicate cage boundaries, double jagged lines indicate openings between cages, single jagged lines indicated openings with doors, and double lines indicate the perch.

Statistical analysis

Female size

A mixed model was used to test for effects of treatment on female size (tarsus length and body weight at day 20 and day 120) and age at fledging, with treatment and mean tarsus length of

the parents as fixed effects, and family and experimenter as a random effect. P-values were considered significant at $P<0.05$ and marginally significant at $P<0.10$.

Experimental song trials

Female location in the experimental set-up was recorded at the beginning of every 15-second interval, for a total of 33 measurements for each of the two 8-minute observation periods (track 1 and track 2) for a maximum possible count of 66 measurements. Cage 3 was considered neutral, and females that did not move from cage 3 for the duration of the trial were removed from the analysis. The end cages (cage 1 and cage 2; cage 4 and cage 5) were pooled, with female location in the experimental set-up interpreted as a preference for song coming from the closest speaker. The average number of counts for females in each group is reported with standard error (SE). Paired t-tests were performed to determine statistical difference between groups. P-values were considered significant at $P<0.05$ (*) and marginally significant at $P<0.10$ (†).

RESULTS

Female Size

All measurements of female size (day 20 weight and tarsus, day 120 weight and tarsus) and fledgling age were not significant different between treatment groups (Figure 5.2; ANOVA: $P>0.10$).

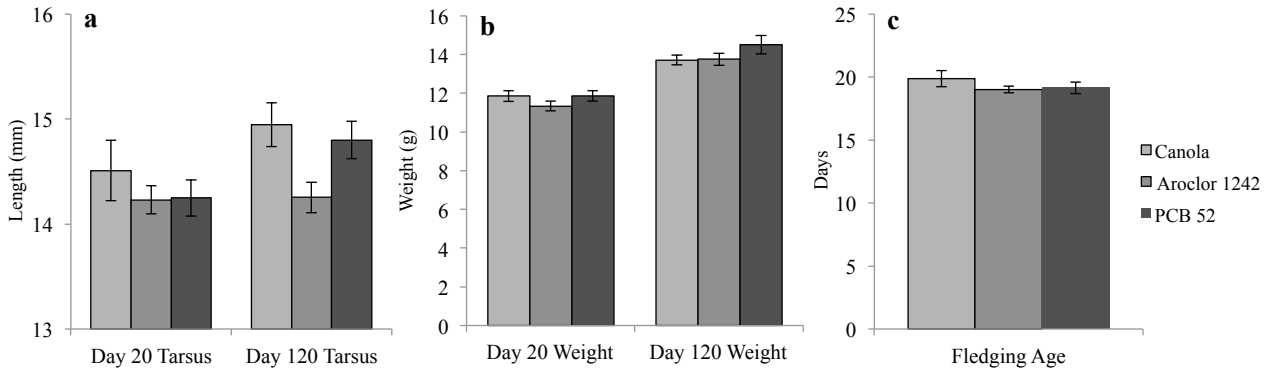

Figure 5.2. Treatment effect on female size and fledging age. **a)** Left tarsus length measured at day 20 and day 120. Bars are mean±SE (ANOVA, $P>0.10$). **b)** Weight measured at day 20 and day 120. Bars are mean±SE (ANOVA, $P>0.10$). **c)** Age at fledging. Bars are mean±SE (ANOVA, $P>0.10$).

Female song preference

Out of the total 265 trials, females made a choice in 228 (86%). When the three female groups were presented with social male song in comparison to isolated male song (Figure 5.3a), only females treated with Aroclor 1242 significantly preferred the social male song (Paired t-test: $P=0.0085$). When the three female groups were presented with the Canola male song in comparison to the Aroclor 1242 male song (Figure 5.3b), females treated with Aroclor 1242 marginally preferred the Aroclor 1242 male song (Paired t-test: $P<0.1$), while all the other groups showed no strong preference of song type. When the three female groups were presented with the Canola male song in comparison to the PCB 52 male song (Figure 5.3c), no females preferred one song type to the other.

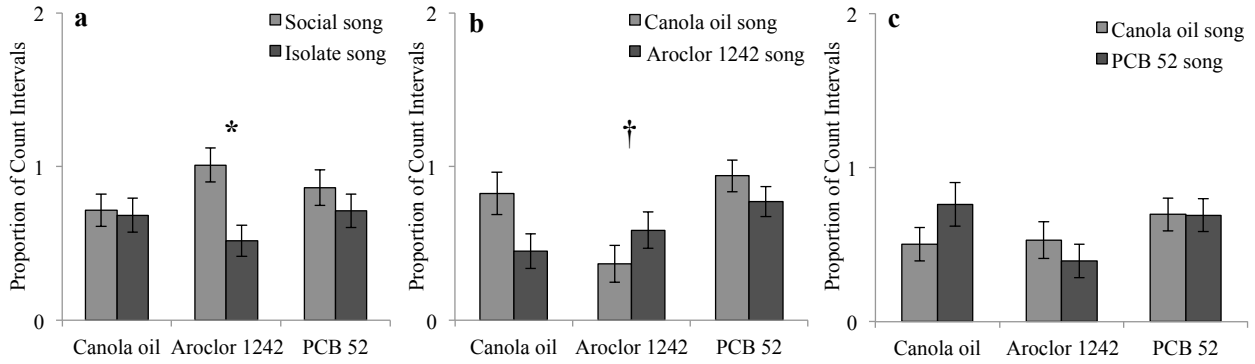

Figure 5.3. Treatment effect on female song preferences. **a)** Preference between social male song and isolate male song from females from each treatment group. Bars are mean±SE (Paired t-test: P=0.0085). **b)** Preference between song from males treated with Canola oil and song from males treated with Aroclor1242 from females from each treatment group. Bars are mean±SE (Paired t-test: P<0.1). **c)** Preference between song from males treated with Canola oil and song from males treated with PCB 52 from females from each treatment group. Bars are mean±SE (Paired t-test: P>0.1).

DISCUSSION

Females who inhabit PCB-contaminated regions in the wild are faced with multiple challenges; not only must they physiologically contend with PCB-exposure from a young age, as mature adults, they also must choose between potential PCB-exposed males, and overcome influences to their own choices that may be compromised due to PCB-exposure. This experiment was designed to elucidate the effects of sublethal PCB-exposure during development on female size, on male song from the females' perspective, as well as investigate the effects of PCBs on female song preference.

The first objective of this experiment was to determine whether female zebra finch size is affected by exposure to sublethal levels of PCBs during development. Results indicate that this PCB exposure during development did not significantly alter adult female weight, tarsus length, or age at fledging (Figure 5.2). The level of PCB exposure used during this experiment was lower than the likely exposure of nestlings in the field (Echols et al. 2004; Maul et al. 2006; McCarty

2002; Secord et al. 1999), therefore it is not surprising that adult female morphological characteristics were not significantly different due to this low PCB exposure.

The second objective of this experiment was to determine whether female zebra finches prefer songs from PCB-treated or untreated males. When control Canola oil females were given the choice of song from a male treated with Canola oil or Aroclor 1242 (Figure 5.3b), they showed no significant preference for either song type. When control Canola oil females were given a song choice from a male treated with Canola oil or PCB 52 (Figure 5.3c), they again showed no significant preference for either song type. Therefore, it is likely that this dosage did alter male song enough to elicit a preference from the control Canola oil females.

The last objective of this experiment was to determine whether females treated with PCBs during development have different song preferences than untreated females. The results show that only females treated with Aroclor 1242 showed significant preference for songs from males raised socially in comparison to males raised in isolation (Figure 5.3a). Females treated with Aroclor 1242 also showed marginally significant preference for songs from males treated with Aroclor 1242 (Figure 5.3b). When the male songs were analyzed for consistency in repetition (citation of thesis: Chapter 3), males treated with Aroclor 1242 had a higher consistency score than the other male treatment groups. Furthermore, these males also had higher spinal density in the HVC than males from other treatment groups (citation of thesis: Chapter 3). Therefore, it is appears that females treated with Aroclor 1242 have a increased discernment for the more consistent male song, perhaps due to altered song center brain morphology as well.

This experiment clearly shows that pure PCB congeners and PCB mixtures do not act the same in biological systems. Although this has been shown in other studies (Bonefeld-Jorgensen et al. 2001; Galassi et al. 2002), it is imperative that toxicology studies in the field continue to look

at congener specific PCB data, and not just total PCB concentrations. Without knowing the specific PCB congeners, it is impossible to correctly predict or understand the impact and effects of exposure.

This experiment also clearly shows that exposure to PCB during development can have important, lasting consequences on adult females. Sublethal, environmentally relevant levels of PCB-exposure during development significantly affected female song preferences. Results from these song preference trials data justify further behavioral assays to disentangle the complicated behavioral consequences of PCB congeners and mixtures administered at different stages of development. Therefore, this study provides another important link in understanding the effects of developmental exposure to environmental PCB contamination.

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Chapter 6:

CONCLUSION

In this thesis I attempted to examine and understand the effects that sublethal levels of PCBs have on passerine song and reproductive behavior. Not only do I believe that the results of this thesis significantly add to the current literature on the behavioral effects of sublethal PCB-exposure, the results of this thesis lead to two important conclusions.

First, the effects on song characteristics and reproductive behaviors in passerines are conditional upon their exposure to specific PCB congeners. The lower chlorinated Aroclor 1242 mixture, and the lower chlorinated pure PCB 52 congener appear to be mimicking the action of estrogens in the avian song system. Because the HVC is the only cerebral song nucleus with estrogen receptors (Fusani & Gahr 2006), and because Aroclor 1242- and PCB 52-exposure causes an increase in dendritic spines only in the HVC and not in the RA as well (chapter 3), it follows that the effect of these PCBs on the song system occurs via estrogen receptor interaction. This interpretation concurs with the results from the only other study on PCBs and birdsong which found that the higher chlorinated mixture of Aroclor 1248 decreased only RA volume (Hoogesteijn et al. 2008). Furthermore, this result is supported in the field by the higher chlorinated PCBs found in black-capped chickadees correlating more inconsistent songs and lower chlorinated PCBs found in song sparrows correlating with higher performance trills (chapter 2). Therefore, it is clear that quantifying the congener-specific PCB profile is vital to understand the possible effects of exposure.

Second, due to the divergent results from differential sublethal PCB congener exposure, and because PCBs are ubiquitous worldwide, results from this thesis highlight the importance of

obtaining congener specific PCB data in behavioral field studies. Environmentally relevant levels of PCB exposure in passerines both in the field and in the laboratory show that PCBs are influencing song and reproductive behavior, key components of reproductive success. Field results show that the congener-specific PCB profiles correlate with song changes in black-capped chickadees and song sparrows (chapter 2). Laboratory exposure to lower chlorinated PCBs, whether pure congeners or a mixture of congeners, results in an increased number of dendritic spines in the HVC, and more consistent song production in adult male zebra finches (chapter 2). Behavioral results from the breeding experiment of adult male zebra finches indicate that PCB-exposed males are less successful (chapter 4), and that females exposed to PCBs during development have stronger song preferences (chapter 5). Therefore, because PCBs are present in organisms at virtually every trophic level and on every continent it is important to consider the effect that low levels of exposure may be having on 'undisturbed' behaviors of wild populations.

In conclusion, results from this thesis significantly add to the body of literature investigating the behavioral effects of sublethal congener-specific PCB exposure and highlight the importance of obtaining congener-specific PCB data in all studies. Because PCB-congeners influence neuronal development and have lasting affects on adult behavior, it is imperative that researchers obtain accurate PCB congener profiles when studying the behavior of organisms in the wild.

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